Developmentally regulated volatiles geosmin and 2-methylisoborneol attract a soil arthropod to *Streptomyces* bacteria promoting spore dispersal

Paul G. Becher[1]*, Vasiliki Verschut[1], Maureen J. Bibb[2], Matthew J. Bush[2], Béla P. Molnár[1,3], Elisabeth Barane[4], Mahmoud M. Al-Bassam[2], Govind Chandra[2], Lijiang Song[5], Gregory L. Challis[5,6], Mark J. Buttner[2], and Klas Flärdh[4]*

[1] Department of Plant Protection Biology, Box 102, Swedish University of Agricultural Sciences, 23053 Alnarp, Sweden

[2] Department of Molecular Microbiology, John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK

[3] Zoology Department, Plant Protection Institute, Centre for Agricultural Research, Budapest, Hungary

[4] Department of Biology, Lund University, Sölvegatan 35, 223 62 Lund, Sweden

[5]Department of Chemistry, University of Warwick, Coventry, CV4 7AL, UK

[6]Department of Biochemistry and Molecular Biology, Monash University, Clayton VIC 3800, Australia.

*corresponding authors:

paul.becher@slu.se

klas.flardh@biol.lu.se

SUMMARY PARAGRAPH

Volatile compounds emitted by bacteria are often sensed by other organisms as odours, but their ecological roles are poorly understood[1,2]. Well-known examples are the soil-smelling terpenoids geosmin and 2-methylisoborneol (2-MIB)[3,4] that humans and various animals sense at extremely low concentrations[5,6]. The conservation of geosmin biosynthesis genes among virtually all species of *Streptomyces* bacteria (and 2-MIB genes in about 50%)[7,8], suggests that the volatiles provide a selective advantage for these soil microbes. We show here that these volatiles mediate interactions of apparent mutual benefit between streptomycetes and springtails (Collembola). In field experiments springtails were attracted to odours emitted by *Streptomyces* colonies. Geosmin and 2-MIB in these odours induce electrophysiological responses in antennae of the model springtail *Folsomia candida*, which is also attracted to both compounds. Moreover, the genes for geosmin and 2-MIB synthases are under the direct control of sporulation-specific transcription factors, constraining emission of the odorants to sporulating colonies. *F. candida* feeds on the *Streptomyces* colonies and disseminates spores both via faecal pellets and through adherence to its hydrophobic cuticle. The results indicate that geosmin and 2-MIB production is an integral part of the sporulation process, completing the *Streptomyces* life cycle by facilitating dispersal of spores by soil arthropods.

MAIN TEXT

One of the most emblematic and abundant microbial volatile organic compounds (VOCs) is geosmin, which is familiar as a characteristic odour of soil[9]. Geosmin is a sesquiterpenoid produced by certain soil microbes, most notably streptomycetes and related actinobacteria, but also by some myxobacteria, cyanobacteria, and filamentous fungi[8]. The enzyme geosmin synthase catalyses the cyclization of farnesyl diphosphate to germacradienol and germacrene D, and then converts germacradienol to geosmin[4]. The gene encoding geosmin synthase is conserved in virtually all sequenced *Streptomyces* genomes (Supplementary Discussion)[7,8]. Streptomycetes are famous for their plethora of specialized metabolites, comprising an enormous range of chemical structures and biological activities, including most of the antibiotics in current clinical use[10]. Each *Streptomyces* isolate has the genetic capacity to produce dozens of specialized metabolites but any given compound is made by only a small percentage of strains. The ubiquity of geosmin production is therefore remarkable and suggests that it confers a selective advantage on the streptomycetes. Here we investigate the biological role of geosmin and find that it is intimately connected to the developmental life cycle of these organisms. Streptomycetes grow vegetatively as mycelial networks that are entangled with the soil particles or other substrates on which they live. The mycelia can spread on a cm-scale by a volatile (trimethylamine)-mediated specialised exploratory mode of growth[11], but dispersal over longer distances occurs through single-celled dormant spores that are formed on specialized aerial hyphae emerging from the surface of colonies[12]. When encountering suitable conditions, spores germinate to give rise to new vegetative mycelia.

Many animals, including humans, can sense geosmin at very low levels, and we perceive it as an off-flavour for example in drinking water and foodstuffs[5]. *Drosophila melanogaster* dedicates an olfactory circuit to the sensing of geosmin, which induces a strong aversive behavioural response in the flies[6,13], and in *Aedes aegypti* mosquitoes it acts as an oviposition cue[14]. However, the advantage of geosmin to the producing microbes has remained unknown.

To investigate possible roles of geosmin and other *Streptomyces* VOCs in the context of soil ecosystems, we asked whether the smell of *Streptomyces* might attract soil-dwelling arthropods. In a network of field traps baited with *Streptomyces coelicolor* colonies, we found significant attraction of springtails (Collembola) compared to control traps (Fig. 1a and c), whereas captures of insects and arachnids were not significantly affected by the *Streptomyces* colonies (Extended Data Fig. 1a and b). Collembola are closely related to insects, but constitute a more basal branch of the Hexapoda (six-legged arthropods)[15]. The attraction of springtails to *Streptomyces* was confirmed in a trapping experiment performed in a tropical greenhouse (Fig. 1b), and could finally be proven by a laboratory Y-tube bioassay with the model springtail *Folsomia candida* (Isotomidae) as test organism (Fig. 1d and e). VOCs emitted by *S. coelicolor* were sufficient to attract *F. candida*.

Next, we recorded chemosensory responses in springtail antennae to identify the VOCs that are sensed by the animals. Odour samples of *S. coelicolor* that were analysed by gas chromatography combined with electrophysiological antennal detection (GC-EAD) revealed that geosmin, its biosynthetic intermediate germacradienol, and the shunt product germacrene D induce sensory responses in *F. candida* (Fig. 2). In addition, the

monoterpene 2-methylisoborneol (2-MIB) also elicits a response. Like geosmin, 2-MIB has an earthy smell, and the genes for 2-MIB biosynthesis are found in approximately half of sequenced *Streptomyces* genomes[7,8]. The sensory detection of geosmin and 2-MIB were further confirmed through electroantennography (EAG) using authentic compounds, which induced dose-dependent responses at 10 and 100 ng (Extended Data Fig. 1c). In Y-tube assays, the springtails showed clear attraction behaviour to geosmin at a dose of 1 ng (Fig. 1f). Further, there was significant attraction towards headspace samples from wild-type *S. coelicolor* (diluted to contain 200 pg of geosmin) but not to headspace samples from a congenic *geoA* mutant (Fig. 1e), and the springtails clearly preferred the wild type over the *geoA* mutant in a choice assay (Fig. 1g). 2-MIB was less active than geosmin and showed no statistically significant attraction when tested in isolation at a dose of 1 ng (Fig. 1f), or at the low dose of 2-MIB present in the diluted extracts of the *geoA* single mutant used in Fig. 1e (21 pg; Extended Data Fig. 2). However, given the choice between a concentration of headspace extract from the geosmin mutant containing 3.2 ng of 2-MIB and a similar extract from the double mutant lacking both geosmin and 2-MIB (Extended Data Fig. 1d-f and Extended Data Fig. 2), *F. candida* preferred the mutant emitting 2-MIB (Fig. 1g), clarifying that both earthy odorants are behaviourally active and serve as attractants for the springtail.

F. candida is mostly known to feed on fungi, but also ingests and digests bacteria, and springtails have been reported to sense volatile chemical signals for localization of food sources[16,17]. In our study, it was obvious that the springtails were feeding on sporulating colonies of *S. coelicolor* (Supplementary Movie 1), and dark grey bacterial biomass was clearly visible in the intestines of the largely transparent *F. candida* (Extended Data Fig. 3a). Also, *S. coelicolor* colonies placed in soil microcosms were grazed upon by *F.*

candida individuals until most of the colony was consumed (Extended Data Fig. 3b). Further, although the prolific specialized metabolism of streptomycetes makes them toxic to some invertebrates[6,18,19], providing *S. coelicolor* as sole food source had positive effects on the survival, moulting and oviposition of *F. candida* springtails (Extended Data Fig. 4). Thus, the ability to sense geosmin and 2-MIB guides springtails to the bacteria as a source of food.

To address the possible significance of the VOCs to the bacteria, we investigated expression of geosmin and 2-MIB biosynthetic genes during the *Streptomyces* developmental life cycle. These studies were conducted with the alternative model species *Streptomyces venezuelae*, which is superior to *S. coelicolor* for the analysis of developmental regulation[12]. In time-resolved global transcriptome analyses, the biosynthetic genes for both geosmin (*geoA*[4]) and 2-MIB (*mibA* and *mibB*, encoding a monoterpene cyclase and a methyl transferase, respectively, that together convert geranyl diphosphate into 2-MIB[3]) were found to be developmentally up-regulated around the time that sporulation is initiated (Fig. 3a). Further, expression of both *geoA* and *mibA-mibB* depended on the regulatory gene *bldM*, encoding a response regulator transcription factor required for development of aerial hyphae and spores (Fig. 3a)[20]. Unexpectedly, the *mibA-mibB* genes were found to form an operon with *eshA* (encoding a putative cyclic nucleotide-binding protein of unclear function[21]) which has the same pattern of expression as *mibA* and *mibB* (Fig. 3a). ChIP-seq analysis showed that BldM directly regulates the promoter of the *eshA-mibA-mibB* operon (Fig. 3b). GC-MS analysis of collected headspace samples showed that production of geosmin and 2-MIB is essentially absent from an *S. venezuelae bldM* mutant (Extended Data Fig. 5).

There was no BldM ChIP-seq signal upstream of *geoA* (Fig. 3b), suggesting that BldM does not regulate *geoA* directly. A survey of other key sporulation regulators revealed that *geoA* also failed to be developmentally upregulated in a *whiH* mutant (Fig. 3a). WhiH is a transcription factor that is required for proper septation of aerial hyphae during spore formation[22]. ChIP-seq analysis showed that WhiH directly regulates the *geoA* promoter (Fig. 3c), and emission of geosmin was strongly reduced in the *whiH* mutant compared to the wild type (Extended Data Fig. 5). Previous studies in *S. coelicolor* showed that *whiH* itself is regulated by another developmental regulator, the RNA polymerase sigma factor encoded by *whiG*[23]. However, expression of *whiH* in *S. venezuelae* is only partially dependent on *whiG*[24]. This observation likely explains why *geoA* is expressed in the *whiG* mutant, although apparently with slight delay compared to the wild type (Fig. 3a). In summary, the production of both earthy odorants is directly coupled to spore formation via transcriptional control of the *geoA* and *eshA-mibA-mibB* loci by key sporulation regulators. These insights provide a mechanistic explanation for the previously reported correlation between geosmin production and sporulation in *Streptomyces*[25,26].

The geosmin and 2-MIB-mediated attraction of springtails and the direct coupling of these VOCs to spore formation suggested that springtails might act as vectors for spore dispersal. Springtails are characterised by anti-adhesive skin patterns and a cuticle covered with hydrophobic layers of wax, leading to a poor adherence of most bacteria to the animal's surface[27,28]. However, we found that 10^4-10^5 spores could be washed off from the surface of *F. candida* individuals that had been exposed to sporulating *S. coelicolor* colonies, and scanning electron microscopy showed that spores adhered to hair-like setae on the *Folsomia* body (seen as short chains of spores in Fig. 4a). Our

findings are in agreement with previously reported adherence of *Streptomyces griseus* spores to the hydrophobic cuticles of springtails and mites[29]. It is likely that the adherence to the unwettable cuticle of the springtails is mediated by the extremely hydrophobic sheath that covers *Streptomyces* spores[12]. To compare adherence of spores and vegetative hyphae, we allowed springtails to feed for 3 days on either *S. coelicolor* wild type strain M145 or the congenic, non-sporulating *whiG* mutant J2400 (a developmental mutant producing only non-sporulating aerial hyphae in addition to the vegetative mycelium). A substantial number of colony-forming units (CFU) adhering to the body surface could be washed off from springtails having been exposed to the sporulating strain, while almost no CFU in form of hyphae were adhering to or could be washed off from springtails exposed to the non-sporulating strain (Extended Data Fig. 6a).

In addition to transport of spores on the body surface, the feeding behavior of the animals offers a second possible route for spore dispersal through defaecation. To test this possibility, we took springtails that had been offered *S. coelicolor* biomass as food, washed off surface-adhered spores, and then collected the faecal pellets that they released. 70.8 % (n=24) of the animals released faecal pellets containing viable *Streptomyces* spores that gave rise to colonies when plated on agar media, confirming that spores were being ingested by the animals and could survive passage through their guts. Springtails that had been fed with the non-sporulating *whiG* strain (containing only vegetative and aerial hyphae but no spores) and thereafter washed, released very few CFU when given time to defaecate, while the sporulating strain dispersed over 100-fold more CFU under these conditions (Extended Data Fig. 6b). Overall, the results show that *Streptomyces* spores are much more efficiently dispersed by *F. candida* than non-

sporulating hyphae, and that dispersal can occur via two different routes: by adherence to the surface of the cuticle and by passage through the gastrointestinal tract.

Finally, using Petri dish bioassays, it was confirmed that spore dispersal by springtails is influenced by the VOCs. We placed the same number of viable spores of *S. coelicolor* wild-type and the geosmin and 2-MIB-deficient double mutant on agar plates and compared to what extent they were dispersed by *F. candida*. The number of spores dispersed and the average distance of their dispersal were positively affected by production of the volatiles in wild-type *S. coelicolor* (Fig. 4b and c). We also complemented the double mutant strain J2192 *in trans* with *geoA* and *mibA-mibB* carried on an integrated plasmid (pIJ10646) and compared it with the same mutant carrying only the empty vector pIJ10770. Complementation restored production of geosmin and 2-MIB and enhanced *F. candida*-mediated dispersal of spores from developed colonies (Extended Data Fig. 6c and d).

In summary, we find in laboratory experiments that the model species *F. candida* senses both geosmin and 2-MIB, and that attraction to the odours mediates spore dispersal for *S. coelicolor* and guides springtails to a source of food. Collembola are dietary generalists that feed on a wide range of microorganisms and even plant material. Numerous studies have shown that microorganisms differ in their quality as food resources for springtails, and springtails discriminate when offered different microbial species as food[30,31]. Two genome sequences show that collembolans have a broad repertoire of enzymes for the degradation of cell wall material, including the peptidoglycan of bacteria[32,33]. Certain microbes likely use structural and chemical defense mechanisms to defend against grazing, for example toxic specialised metabolites[19,34,35]. Streptomycetes and other

geosmin-emitting microbes often produce such metabolites that are toxic to invertebrates[10,18,36]. A recent study showed that *Caenorhabditis elegans* avoids toxin-producing streptomycetes by sensing and escaping from small molecules released by these bacteria[19]. Further, the strong aversion of *Drosophila* flies towards geosmin was speculated to help them avoid contaminated and potentially poisonous food sources[6]. It is therefore interesting that the *F. candida* springtails were not negatively affected when feeding on *S. coelicolor* as the only source of food.

Springtails are adapted to exposure to various toxic organic substances and xenobiotics in the soil, and their genomes contain expanded gene families implicated in detoxification mechanisms[32,33], presumably giving them the capacity to tolerate specialized metabolites produced by streptomycetes. Interestingly, exposure to *Streptomyces* also stimulated ecdysis (Extended Data Fig. 4b), which might be seen as a growth response to the availability of food, or act as a detoxification mechanism[37]. It seems likely that such mechanisms allow springtails to feed on streptomycetes, a resource that can be toxic to other organisms, such as nematodes[19]. In addition, apart from being food, *Streptomyces* spp. as producers of antibiotics might play a role in protecting springtails from pathogens, comparable to the relation described between *Streptomyces* and soil-dwelling beewolf larvae[38].

The *Streptomyces* genus is of an early origin[39], and the broad distribution of geosmin and 2-MIB production among streptomycetes indicates that both traits are ancestral. Interestingly, while *Drosophila melanogaster* uses an olfactory receptor (OR) to detect geosmin, Collembola, which separated from insects over 450 million years ago[15], use a different chemoreceptor, which must be of earlier type since ORs and their associated co-receptor Orco evolved relatively late in insect evolution and are not found in Collembola

(Supplementary Discussion) and other basal hexapods[40,41]. Overall, the VOC-mediated interaction between streptomycetes and springtails that we describe here is likely to be ancient.

Our laboratory experiments suggest that streptomycetes benefit from emitting geosmin and 2-MIB as part of their developmental programme because these volatile scents guide springtails to sporulating microcolonies, and the animals serve as vectors for spore dispersal. The top layers of soil, where both streptomycetes and springtails are abundant, form a heterogenous and highly structured matrix, which may impede long-distance spore transport by water and wind. Vectoring by soil arthropods should provide a beneficial mode of dispersal in such belowground environments, and contribute to shaping the microbial communities therein[42]. Undoubtedly, chemical information in the soil ecosystem is not limited to geosmin and 2-MIB, and other volatiles that may act as either attractants or repellents, such as oxylipins, need to be considered for a comprehensive understanding of chemical ecological and specific trophic interactions between soil organisms[43]. Thus, the relevance of the *Streptomyces*-produced geosmin and 2-MIB in the interactions between the bacteria and springtails should be further tested in a community context.

METHODS

Bacterial strains, plasmids, oligonucleotides and growth media. Strains, plasmids and oligonucleotides used in this study are described in Supplementary Table 1. *Escherichia coli* strain DH5α was used for plasmid and cosmid propagation. *E. coli* strain BW25113 containing a λRed plasmid, pIJ790, was used to create disrupted cosmids[44]. Cosmids and plasmids were conjugated from the *dam dcm hsdS E. coli* strain ET12567 containing pUZ8002, as described previously[45-47]. Strains of *S. coelicolor* were cultivated on soya flour mannitol agar medium (SFM) and spores were prepared, as described previously[47]. Strains of *S. venezuelae* were cultivated on maltose yeast extract medium (MYM), as described by Bush *et al*.[48].

Construction of *S. coelicolor and S. venezuelae* mutants. Using 'Redirect' PCR targeting[45,46], an *S. venezuelae whiH* mutant was generated in which the *whiH* coding sequence was replaced with an apramycin resistance (*apr*) cassette. Cosmid 1D05 was introduced into *E. coli* BW25113 containing pIJ790 and the *whiH* gene (*vnz27205*) was replaced with the *apr-oriT* cassette amplified from pIJ773 using the primer pairs *whiH*_DEL_F and *whiH*_DEL_R. The resulting construct was introduced into wild-type *S. venezuelae* by conjugation and null mutant derivatives, generated by double crossing over, were identified by their apramycin-resistant, kanamycin-sensitive and morphological phenotypes. A representative *whiH* null mutant was designated SV8.

The *S. coelicolor* Δ(*mibA-mibB)::apr* mutation was generated via the same approach, using λRed-mediated recombination to modify the 6D11 cosmid that carries the *mibA* and *mibB* genes (*sco7700-7701*). The *mibA* and *mibB* genes were replaced with a single *apr-oriT* cassette amplified from pIJ773 using the primer pairs *mib*_DEL_F and *mib*_DEL_R.

The resulting mutant allele was introduced into the chromosome of the unmarked Δ*geoA* mutant of *S. coelicolor* (J3003)[45], and a representative Δ*geoA* Δ(*mibA-mibB)::apr* double mutant was designated J2192.

For *in trans* complementation of *geoA* and *mibAB* mutations, the *eshA-mibA-mibB* region was amplified from *S. coelicolor* genomic DNA with primers mib_F and mib_R and cloned between the HindIII and AvrII sites in the integrating vector pIJ10770, the *geoA* gene was amplified with primers geoA_F and geoA_R and cloned in the EcoRV site of the same plasmid, resulting in plasmid pIJ10646.

Trap networks in the field. Sticky traps were used to test the attraction of soil arthropods to live cultures of *S. coelicolor* (strain M145, grown on SFM agar) at two woodland sites at Alnarp, Sweden (55°39'38.0"N 13°04'36.8"E). Both sites were divided into 5 plots and each plot received all treatments (sticky traps baited with *S. coelicolor* or controls). Cell cultures of *S. coelicolor* were prepared by streaking approximately 7 x 10^4 CFU onto Petri dishes (3.5 cm diameter) with SFM agar. Plates were incubated in darkness at 27°C for 8 days before use in the traps. As control treatments traps baited with the substrate only (SFM agar) and unbaited traps were used. Petri dishes of the bacterial cultures or controls were uncovered and placed in the centre of white sticky traps (glue boards of 16 x 9 cm, Silvandersson, Knäred, Sweden) onto the ground of the study sites. A random number generator was used to decide upon the location of the treatments in the plots. After 24 hours the traps were collected and the number of captured insects, springtails and arachnids were counted. The experiment was repeated once more at the following day (resulting in 20 traps per treatment in total).

The attraction of springtails to cultures of *S. coelicolor* was also evaluated in a glasshouse with beds containing tropical plants, at the Swedish University of Agricultural Sciences, Alnarp. The same type of traps was used as described for the field experiment, with unbaited traps as control. The glasshouse was subdivided in 5 plots, with each plot receiving 5 replicates of bacterial baited trap and control. The traps were collected 4 days after their placement and the number of springtail captures was recorded. The experiment was repeated 4 times in consecutive dates, resulting in 100 traps per treatment in total.

Generalized linear mixed models (GLMMs) were used to analyse the effect of trap bait on the captures of springtails, insects, and arachnids from the field experiment. A Negative binomial distribution was used to correct for overdispersion of data when initially analysed with a Poisson distribution[49]. Models included treatment as the explanatory variable (*S. coelicolor*, agar substrate and unbaited traps). The site and date of collection were included as random factors. Similarly, we also analysed the data from the glasshouse experiment using GLMMs, with negative binomial distribution. Treatment was included as the explanatory variable (*S. coelicolor* and unbaited traps), while plot and date were added to the models as random factors. Significance of the explanatory variable was tested with type II Wald x^2-statistics. A Bonferroni correction was applied when pairwise comparisons were performed. All analyses were carried out in R (v. 3.3.3; R Foundation for Statistical Computing, Vienna, AT) with the packages lme4, MASS and multcomp.

Collembola. We established a rearing of the springtail *Folsomia candida* Willem (Terra-Jungle, Cologne, Germany). Identity of the species was confirmed by sequencing of the cytochrome c oxidase subunit 1 (COI) showing 99% sequence similarity with *F. candida*

in a BLAST search on the NCBI database. Few experiments (tests involving non-sporulating *whiG* strain or complemented double mutant strain J2192) were performed with a different colony of *F. candida* originating from a laboratory culture at Aarhus University, Denmark[50]. The rearing was maintained in darkness at 20°C, on a mixture of gypsum plaster and charcoal (Sigma) (9:1, v/v), kept in sealed Petri dishes. Once a week a pinch of semi-artificial springtail food (Terra-Jungle) was added to the dishes, while distilled water was added every second day to retain moist. Preliminary experiments indicated that *F. candida* was behaviourally more active when previously kept in a soil substrate. Springtails used for behavioural assays therefore were kept on commercial soil substrate (Kronmull, Weibull Trädgård AB, Hammenhög, Sweden) in high Petri dishes (6·5 cm high and 12 cm diameter) and transferred to gypsum-charcoal plaster without food 24 h prior testing.

Volatile collections. Open plates of *S. coelicolor* cultures were individually enclosed in 500-mL PET cooking bags (Toppits, Klippan, Sweden). A stream of charcoal-filtered air (50 mL min^{-1}) was pulled over the headspace of the bacterial cultures, leaving the bag through an air filter adsorbing the emitted bacterial volatile compounds. Filters were made of glass tubes (40 mm length, 0.3 mm ID) containing 35 mg of Porapak Q (80/100 mesh, Altech, Deerfield, IL, USA) held between glass wool plugs. Before sampling, filters were rinsed with 2 mL of redistilled methanol and 2 mL of redistilled heptane. Volatiles were collected for 23 h at 22°C ± 2ºC and then eluted from the filters with 0.8 mL of redistilled heptane. For normalization of quantitative chemical analyses, the bacterial biomass, grown on agar medium covered with a cellophane membrane, was scraped off from the agar medium and the dry weight was determined after drying overnight at 80°C. Moreover, heptyl acetate was added as internal standard for

quantification of metabolites produced by mutants but not applied for material tested in the bioassays.

Chemical analysis. Headspace collections were analysed by coupled gas chromatography-mass spectrometry (GC-MS; 6890 GC and 5975 MS; Agilent Technologies, Palo Alto, CA, USA), operated in the electron impact ionization mode at 70 eV. The GC was equipped with a HP-5MS (Agilent Technologies Inc.) fused silica capillary column (60 m x 0.25 mm; d_f = 0.25 μm). Helium was used as the carrier gas at an average linear flow rate of 35 cm s^{-1}. Two microliters of each sample were injected (splitless mode, 30 s, injector temperature 225°C). The GC oven temperature was programmed from 50°C (2 min hold) at 8°C min^{-1} to 230°C (10 min hold). The transfer line between the GC and MS was programmed to hold at 150°C and to track in synchrony with the GC oven above that temperature. Compounds were tentatively identified by matching their mass spectra with those in the MS Libraries (NIST 11, Wiley) using the software ChemStation (MSD Chemstation D.01.02.16 Agilent Technologies) and further verified by co-injection of reference compounds (except germacradienol) and comparison to published Kovats retention index (KI) values and mass spectra[51-54].

Electroantennography. Coupled gas chromatographic-electroantennographic detection analysis (GC-EAD) was performed to identify the key bacterial odour components that elicit antennal responses in *F. candida*. Agilent 6890N gas chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA), equipped with a HP-5 capillary column (30 m × 0.32 mm, d_f = 0.25 μm, J&W Scientific, Folsom, CA, USA), was used with on-column injection mode. The oven temperature was programmed as follows: 50°C for 1 min, then

15°C min^{-1} up to 230 °C and 5 min isothermal. Helium was the carrier gas at constant flow rate of 45 cm s^{-1}. The GC effluent was split equally (Gerstel Graphpack 3D/2 crosspiece) in half allowing simultaneous signal detection at a FID (280°C) and a heated EAD port (220°C) (transfer line: Syntech, Kirchzarten, Germany). At the EAD port, the capillary effluent was delivered to the antennal preparation in a stream of charcoal-filtered and humidified air (1 L min^{-1}) in a glass tube (150 mm x 8 mm). The head of randomly selected adult female *F. candida* was excised and inserted into a pulled glass capillary (ID 1.17 mm, Syntech) filled with Ringer solution[55] and attached to a reference silver/silver chloride electrode held in a micromanipulator (MP-15, Syntech). The antennal signal was amplified 10 times, converted to a digital signal by a high input impedance DC amplifier interface (IDAC-232, Syntech) and recorded simultaneously with the FID signal using a GC-EAD software (GC-EAD 2000, version 1.2.3, Syntech). For every recording a new antennal preparation was used and 2 µL of *S. coelicolor* strain M145 volatile collection extract was injected to the GC. In total six GC-EAD recordings were averaged to analyse for consistent antennal responses. The GC-EAD active compounds were also compared with authentic standards. The quantity of each compound was calculated on the basis of the peak area and calibrated by comparison with decyl acetate as internal standard.

Antennal responses of geosmin and 2-MIB from bacterial headspace collections were further verified by electroantennography (EAG), puffing ng-amounts of authentic compounds onto antennae. Geosmin and 2-MIB were applied at 10 and 100 ng in n-hexane on a filter paper disk (12.7 mm Ø; Schleicher & Schnell GmbH, Dassel, Germany), which was then placed into a Pasteur-pipette. The blank (empty filter paper), solvent blank (filter with *n*-hexane) and the test compound stimuli were randomized and

tested in 8 replicates on female antennae. The stimulation time was 0.5 s followed by a one-minute recovery period. For the analysis the same instrument, odour delivery system and mounting technique was used as described above. EAG amplitudes to the tested compounds were log-transformed, to meet the assumptions for parametric tests and analysed using linear mixed effects models. Models included stimulus as the explanatory variable, while antenna was added as a random factor. Tukey's post hoc test was used to perform pairwise comparisons within the different stimuli, with a Bonferroni correction. Wald tests were carried out to test the significance of the explanatory variable, while all statistical procedures were conducted using R packages lme4, MASS, car and multcomp.

Y-tube olfactometer assay. Before testing, springtails were starved for 24 h in new Petri dishes lined with the mixture of gypsum plaster and charcoal (9:1, v/v), moistened with distilled water. Springtails were tested for attraction to volatile collections of different *S. coelicolor* strains, geosmin and 2-MIB in a Y-shaped olfactometer (schematic drawing in Fig. 1d) made of glass tubing (8 mm ID) designed on the basis of a similar type of olfactometer described by Bengtsson *et al.*[56]. The olfactometer had a 30 mm long base and 40 mm long arms (in an angle of 45°). Each side arm was connected to a glass cylinder (60 mm long, 20 mm ID), which served as odour release compartment. Both arms were connected to a charcoal filter. The base of the Y-tube was connected with Teflon tubing to a flow meter and then to a suction pump generating a flow of filtered air (12 mL min^{-1}) through the two arms, with the odour-release compartments to the base. A piece of gauze was interjected between the tip of the base and the Teflon tubing to prevent animals from escaping. On the top, at a distance of 1.5 cm from the base end, the olfactometer had a hole of 3 mm ID through which springtails were introduced. After introducing a single springtail, the hole was closed with a Teflon plug. The position of

the springtail was recorded for 10 min or until the springtail entered into a side arm, which was counted as response. Assays were performed in a climate chamber at 25°C and 60% ± 5% RH, under diffuse dim light (5 lux). All headspace samples and geosmin were diluted in redistilled heptane, while 2-MIB was diluted in redistilled hexane. Headspace samples were diluted to certain concentrations of geosmin, 2-MIB or chalcogran (used to equilibrate samples of the *ΔgeoA ΔmibAB* mutant) and tested at the doses shown in Extended Data Fig. 2. Five microliter of odour sample or solvent (heptane or hexane) as a control were loaded onto filter papers (1 cm^2) and inserted into the odour release chambers. Each day the arms delivering the odour samples or control were switched and each olfactometer was used for only 60 min (approx. 5-7 replicates). The total number of replicates for each test is given in Extended Data Fig. 2. Odour attraction of springtails was analysed by an exact binomial test for the hypothesis that the true probability of success equals 0.5.

Performance tests of *F. candida*. Survival was measured for individual *F. candida* adults, which were kept for 10 days in glass tubes (1.6 x 10 cm) sealed with metal lids (Kapsenberg caps) containing 1-week-old cultures of *S. coelicolor* strain M145 covering completely the SFM agar medium (inoculation with 6.6 x 10^7 CFU; n=31). All springtails were kept without food for 24 h prior experimentation. A control group was tested in glass tubes with pure SFM agar medium (n=23). To reduce development of microbial contamination in the control treatment the springtails were transferred every third day to glass tubes with fresh SFM medium. Preliminary experimentation revealed no mortality of springtails due to the transfer process. In addition to survival, the time of ecdysis, the time of oviposition, and the total number of eggs produced per adult were also recorded and analyzed. Average survival, oviposition and ecdysis times were calculated and the

treatment vs control was compared by the Kaplan-Meier survival analysis[57]. Average numbers of eggs were compared by Likelihood Ratio tests produced by (GLM) procedures, with a negative binomial distribution, for a level of significance α=0.05, while all statistical procedures were conducted with R packages MASS, car and survival.

RNA isolation and DNA microarray analysis. RNA isolation and microarray transcriptional profiling of the *S. venezuelae* developmental RNA time courses were performed as described previously[58,59]. The resulting data were processed as described by Bush *et al*.[48].

Chromatin immunoprecipitation, library construction, sequencing and ChIP-seq data analysis. For WhiH, wild-type *S. venezuelae* (ATCC 10712) and its derivative SV8-pIJ6793 (*whiH::apr attBΦBT1::whiH-3xFLAG*) were grown in MYM liquid sporulation medium and ChIP was conducted using anti-FLAG M2 gel suspension (Sigma-Aldrich A2220), as described previously[48]. For BldM, wild-type *S. venezuelae* and its congenic *bldM* null mutant (SV13) were grown in the same way, and ChIP was conducted using an anti-BldM polyclonal antibody, as described previously[20]. Library construction and sequencing were performed as described previously[48] by The Earlham Institute, Norwich Research Park Norwich, UK. The ChIP-seq data were analyzed as described by Bush *et al*., 2013[48].

Spore vectoring, feeding, and dispersal assays. We investigated the possible mode of spore dispersal by *F. candida*. First, we studied if springtails transmit spores via their outer body. Two groups of *F. candida* adults, kept without food for 24 h prior experimentation, were exposed for 1 h at 25°C to sporulating cultures of *S. coelicolor* on

SFM substrate. Individuals of the first group were used for scanning electron microscopy. The animals were immersed in aceton for 15 minutes and then left for air drying. The dried samples were carefully glued onto SEM stubs, and sputter-coated with gold (Cesington 108 auto, 45 seconds, 20mA). The preparations were examined using a scanning electron microscope (SEM; Hitachi SU3500) at 5 kV. Individuals of the second group (10 replicates) were transferred singly to 50 µL of 0.05% Tween-20 and vortexed gently for 30 s to obtain suspensions of spores that were washed off the springtail bodies[60]. The suspensions were diluted, plated on SFM substrate and incubated to estimate the number of spores (CFU) that were attached to the body surface of the springtails.

Next, we tested if springtails disperse viable spores through their faeces. *S. coelicolor* cultures were grown on SFM medium covered with cellophane membranes[47] to provide springtails with sporulating *S. coelicolor* biomass free from remnants of nutrient agar. From sporulated *S. coelicolor* cultures (8 days incubation at 27°C), the bacterial biomass was scraped off the cellophane surface using an inoculation loop. Groups of *F. candida* adults previously kept without food for 24 h were transferred onto a Petri dish lined with plaster and provided with a wad of *S. coelicolor* biomass corresponding to a quarter of the culture from a cellophane disk (providing ad libitum biomass to feed upon). Springtails were left to feed on the bacteria for 48 h. As *F. candida* is whitish and to large extent transparent, feeding activity could be confirmed by a colour change of their intestines to dark grey of the bacterial biomass (Extended Data Fig. 3a). After the feeding period, single springtails were transferred to 1.5 mL Eppendorf tubes containing 50 µL of 0.05% Tween-20 and vortexed gently for 30 s in order to remove spores from their cuticle[60]. No significant number of spores was detected after repeated Tween-20

washings, showing that the washing procedure was efficient. The springtails were then transferred individually into Petri dishes (3.5 cm diameter) lined with SFM agar and incubated for 3 h at 20°C in darkness. Subsequently faecal pellets produced by each individual were collected and smeared onto new SFM agar plate. After incubation period for 8 d at 27°C, colonies of *S. coelicolor* deriving from the faecal pellets were recorded.

To compare dispersal of a sporulating to a non-sporulating strain, *S. coelicolor* strain M145 and the congenic *whiG* mutant strain J2400 were cultivated on SFM medium for 10 days. Both strains had formed dense lawn of mycelium, but only strain M145 had formed spores while strain J2400 only produced vegetative and aerial hyphae. A 2x2 cm^2 piece of the mycelial lawn of each strain was scraped off and used to feed 12 springtails (starved for 24 hours) for 3 days in a plaster-lined Petri dish. Each springtail was then washed in 0.05% Tween-20 as described before. The washing liquid was plated on SFM medium to determine number of CFU washed off per animal. The washed springtails were then kept individually on mannitol minimal medium agar for 20 hours to allow time for defaecation. Springtails were then removed and the number of excreted CFU per plate that formed upon incubation at 30°C was determined. Numbers of CFU originating from treatment with sporulating and non-sporulating strains were compared by Mann-Whitney test.

Furthermore, we monitored grazing by *F. candida* on *S. coelicolor* in soil microcosms. *S. coelicolor* strain J2192/pJ10646 was grown to a sporulating lawn on mannitol minimal medium, and agar plugs with bacterial lawn on surface were excised and placed in 18 g autoclaved potting soil (Weibulls, Sweden) in plastic cups (9 cm diameter). Ten *F. candida* individuals were added to the soil of one set of cups, and no springtails to the

other. Appearance of the bacterial lawn was monitored and photographed after 6 days of incubation at room temperature.

Finally, we monitored the effect of geosmin and 2-MIB emitted by *S. coelicolor* bacteria on spore dispersal by springtails. A droplet (5 µl) of spore suspension (5 x 10^8 CFU mL^{-1}) of either *S. coelicolor* M145 (wild type) or J2192 (*ΔgeoA ΔmibAB*) was added at a distance of 3.5 cm from the centre onto a Petri dish (9 cm diameter) containing SFM agar. The droplet was left to soak into the agar for 30 min to form a round film of spores on the surface of the SFM agar. Three *F. candida* adults that were kept without food for 24 hours were transferred to the centre of the dish and left to move freely for 30 min, in dark at room temperature. Springtails were then removed and plates were incubated at 27°C for 5 days. Each treatment was replicated 25 times and after the incubation period the number of newly formed colonies (not contacting the area of the initial 5-µl inoculum) and the distance between inoculum and the farthest newly formed colony were measured. For the comparison between different treatments General Linear Model (GLM) procedures were used to produce Analysis of Deviance for both the number of newly formed colonies and the distance, with a normal distribution for distance data and quasi-Poisson distribution to correct for overdispersion for the number of newly formed colonies data. Data corresponding to the distance were initially analyzed with Bartlett's test to check for homogeneity of variances, while normality of the residuals and the Q-Q plots were checked through visual inspection.

To determine whether *in trans* complementation with *geoA* and *mibA-mibB* improved the ability of the double mutant strain J2192 to be dispersed by springtails in agar plate assays, strain J2192/pIJ10770 (carrying an integrated empty vector) and J2192/pIJ10646

(with same vector carrying *geoA* and *eshA-mibA-mibB* genes) were inoculated with same amount of spores (5×10^3) in a 10 μL drop on mannitol minimal medium agar plates and incubated to allow formation of sporulated patch in the middle of the plate. The two strains were found to produce similar amounts of viable spores in such patches, and harvested spores of the strains germinated with the similar efficiency (77 +/- 20% and 74 +/- 17% viability, respectively). To each plate 2 starved springtails were added and allowed to roam for 2 hours, before they were removed. Plates were incubated for 8 days to allow new colonies to develop, and number of dispersed colonies per plate were determined and compared by Mann-Whitney test.

DATA AVAILABILITY STATEMENT

The transcriptional profiling data from Affymetrix arrays and the ChIP-Seq data in Fig. 3 have been deposited at the ArrayExpress Archive of Functional Genomics Data (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-5853 for the *whiH* mutant array data, E-MTAB-2716 for the *bldM* mutant array data, E-MTAB-6702 for the WhiH ChIP-Seq data, and E-MTAB-2698 for the BldM ChIP-Seq data). Source data for Figs. 1a,b,e,f,g and 4b,c and Extended Data Figs. 1a,b,c, 4, 5 and 6a,b,c are included in this article and its Supplementary Information files. Other data that support the findings of this study are available from the corresponding authors upon reasonable request.

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CORRESPONDING AUTHORS

Correspondence and requests for materials should be addressed to Paul G. Becher and Klas Flärdh.

ACKNOWLEDGEMENTS

We thank Ola Gustafsson for assistance with electron microscopy, Martin Holmstrup for helping us to establish *F. candida*, Maria Karlsson for confirming species identity of *F. candida*, Thomas Verschut for discussions, Göran Birgersson for technical support, statistics@slu and Friedrich Jüttner for comments on the manuscript. This work was funded by a PlantLink seed money grant to PGB and KF, BBSRC grants BB/H006125/1 and BB/I002197/1 to MJBu, BBSRC Institute Strategic Programme Grants BB/J004561/1 and BB/P012523/1 to the John Innes Centre, grants from the Crafoord Foundation and support through the SLU Centre for Biological Control to PGB, and grants from the Swedish Research Council (2010-4463 and 2015-05452) to KF. BPM was supported by the New National Excellence Program of the Ministry of Human Capacities (ÚNKP-19-4) and by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.

AUTHOR CONTRIBUTIONS

MJBut, PGB and KF designed the research. VV performed the field trap experiments and statistical analyses. VV and PGB did the work involving springtails and analyses of volatiles, and performed together with EBa, and KF the spore dispersal assays. BPM performed the GC-EAD experiments. MJBi and MMA-B performed the time-resolved transcriptional profiling, MJBi, MJBus and MMA-B performed the ChIP-seq experiments, and GC analyzed the ChIP-seq data. MJBi and MJBus constructed mutants

and plasmids. EBa and KF identified WhiH as a regulator of *geoA*. LS and GLC performed initial GC-MS analyses of the *Streptomyces* mutants. PGB and KF wrote the paper together with VV and MJBut. All authors discussed results and commented on the manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

FIGURE LEGENDS

Figure 1

Attraction of springtails to *Streptomyces* headspace, geosmin or 2-methylisoborneol. **a**, Number of springtails captured with sticky traps baited with wild-type *S. coelicolor* strain M145, compared to control traps that were kept without bait or held a non-inoculated plate of culture medium (n=19) in a 1-day field experiment. **b**, Number of springtails captured with sticky traps baited with *S. coelicolor* strain M145 (n=93) compared to unbaited traps (n=100) in a glasshouse experiment over a period of 4 days. Traps baited with *S. coelicolor* attracted significantly more springtails in the field (**a**, $X^2_{2, 57}$ =19.389, P<0.001) as well as in the glasshouse (**b**, $X^2_{1, 193}$ =6.049, P=0.014) than control traps. The boxplots show medians, upper and lower quartiles as well as the span of data points that are within 1.5 times the interquartile range. **c**, Schematic drawing of sticky trap with bait. **d**, Schematic drawing of Y-tube setup. **e-g**, Odour-mediated attraction of the springtail *F. candida* tested in a Y-tube. Headspace volatiles of *S. coelicolor* strain M145 (0.2 ng geosmin, 0.021 ng 2-MIB) induced significant attraction of *F. candida* (**e**, P<0.001 **) whereas the equivalent headspace of the geosmin-deficient mutant J3003 (*ΔgeoA*; 0.021 ng 2-MIB) was not attractive (**e**, P=0.69, ns). Accordingly, geosmin (1 ng) alone was sufficient to attract *F. candida* (**f**, P<0.001 **) and *F. candida* preferred *S. coelicolor* M145 to the *ΔgeoA* mutant when having the choice between the two strains (**g**, P=0.024 *). Attraction to 1 ng of 2-MIB was not significant (**f**, P=0.061, ns) but absence of 2-MIB reduced attraction (**g**, P<0.001 **) when headspace of the double-mutant J2192 (*ΔgeoA ΔmibAB*) was tested against the equivalent headspace from J3003 (*ΔgeoA*, 3.15 ng 2-MIB, which is 150x concentration as compared to **e**). For each treatment, preference for one of the two arms of the Y-tube was examined by an exact binomial test. The bars show the ratios of attraction of springtails to the two arms of the

Y-tube. Error bars show the 95% confidence intervals (C.I.). Equal volumes of the corresponding solvent (double distilled heptane or hexane) were offered as control in **e** and **f**.

Figure 2

Antennal responses to *Streptomyces* headspace components in *F. candida*. Gas chromatogram of sampled *S. coelicolor* volatiles (upper trace) and electroantennogram (lower trace) showing the mean responses (n=6) of *F. candida* springtail antennae towards the volatiles eluting from a HP-5 column of a gas chromatograph (GC). The sketch illustrates the GC with flame ionization detector (FID) and coupled electroantennographic detection (EAD). The inserted photograph shows an immobilized female of *F. candida* fixed into a glass capillary having a precise head dissection for antennal recording.

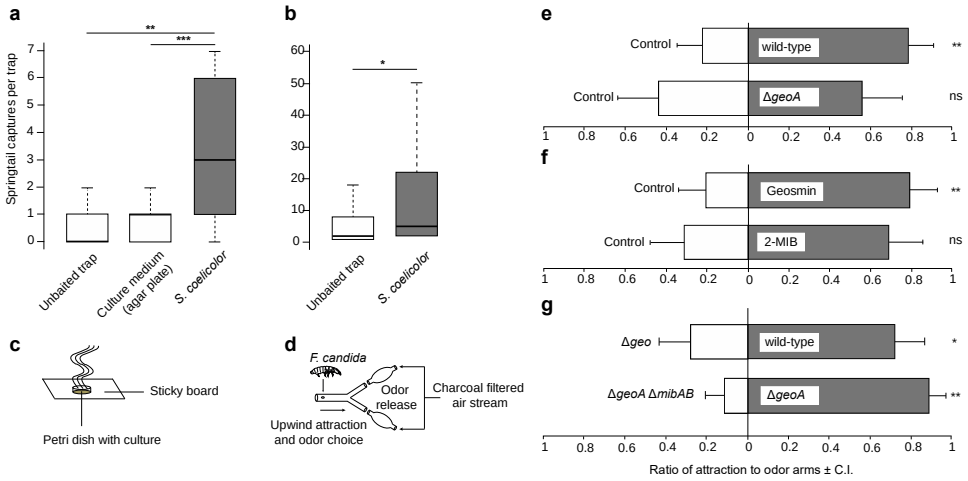
Figure 3

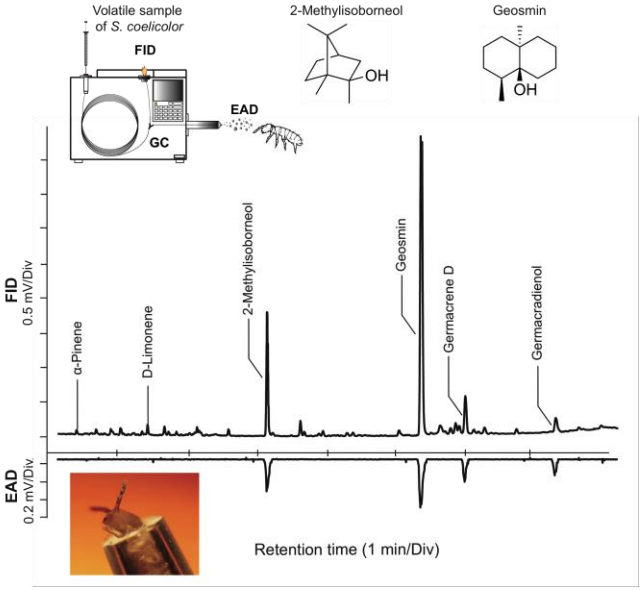
Developmental regulation of the geosmin and 2-MIB biosynthetic genes. a, Microarray transcriptional profiling data for *geoA*, encoding geosmin synthase (upper panels) and the three co-transcribed genes *eshA*, *mibA*, and *mibB* (*eshA* in blue, *mibA* in red, and *mibB* in green) (lower panels; the latter two genes encode the 2-MIB biosynthetic enzymes), during submerged sporulation in wild-type *S. venezuelae* (wt) and congenic mutants lacking the key regulators of sporulation *bldM* (strain SV13), *whiA* (strain SV11), *whiB* (strain SV7), *whiG* (strain SV6), *whiH* (strain SV8), and *whiI* (strain SV10). In each panel, the x axis indicates the age of the culture in hours, and the y axis indicates the per-gene normalized transcript abundance ($\log_2$), based on three independent cultures. For the wild type, 10 to 14 h corresponds to vegetative growth, 14

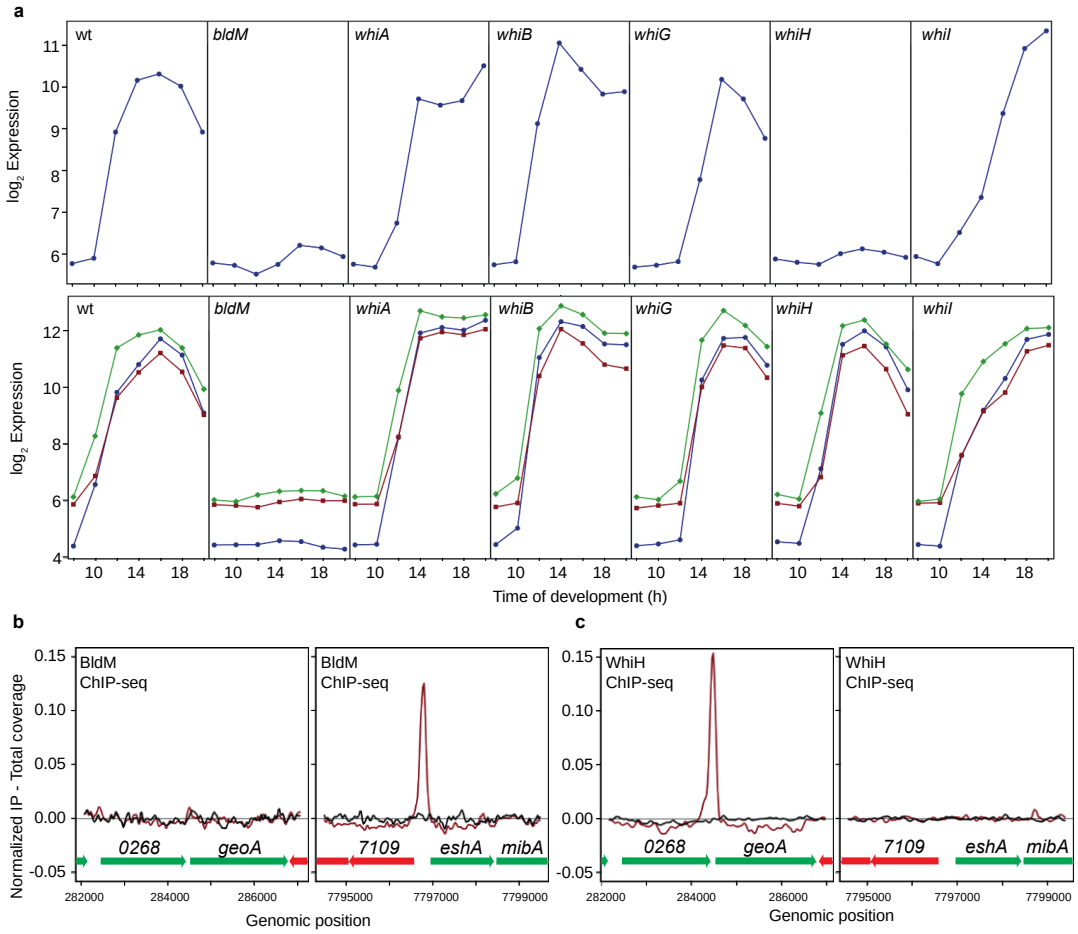
to 16 h corresponds to the onset of sporulation (fragmentation), and 16 h onwards corresponds to sporulation. **b**, BldM binding to the 2-MIB biosynthetic locus (right panel). Anti-BldM polyclonal ChIP-seq data for wt *S. venezuelae* are shown in brown and anti-BldM polyclonal ChIP-seq data for the Δ*bldM* control strain are shown in black. The equivalent data for the *geoA* locus are shown as a negative control (left panel). **c**, WhiH binding to the *geoA* locus (left panel). Anti-FLAG ChIP-seq data for the strain expressing a functional C-terminally 3xFLAG-tagged WhiH are shown in brown and anti-FLAG ChIP-seq data for the control strain (wt *S. venezuelae*) are shown in black. The equivalent data for the *eshA-mibA-mibB* locus are shown as a negative control (right panel). Genes running left to right are shown in green, and genes running right to left are shown in red.

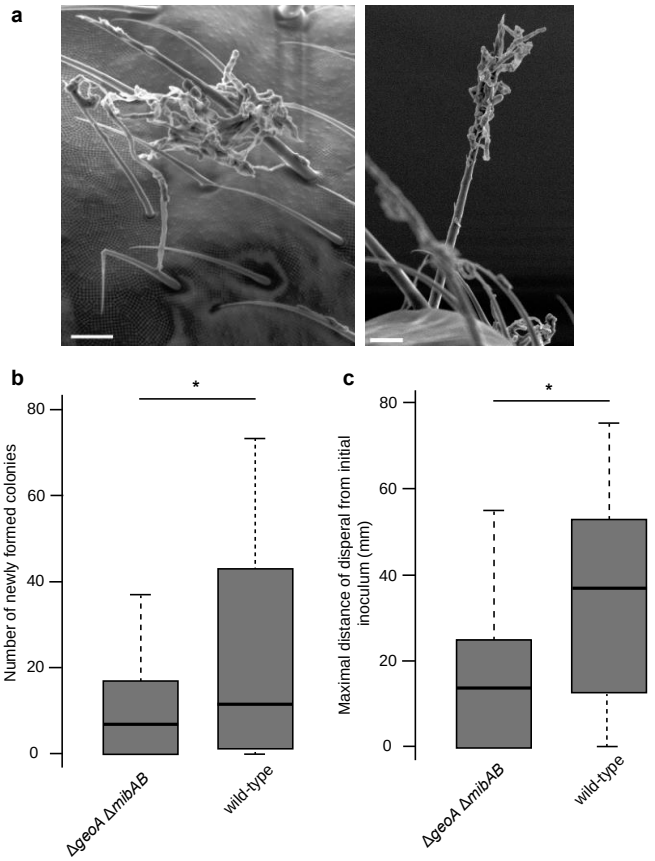
Figure 4

Dispersal of *S. coelicolor* spores mediated by springtails. a, Adherence of short chains of spores to setae on springtails that had been exposed to sporulating *S. coelicolor* culture, visualised by scanning electron microscopy. Representative results from two independent experiments are shown. Size bars, 5 μm. **b**, **c,** Effect of geosmin and 2-MIB on springtail-mediated spore dispersal from *S. coelicolor* colonies. The presence of geosmin and 2-MIB in *S. coelicolor* strain M145 (wt; n=24) colonies resulted in higher numbers of newly formed colonies due to springtail dispersal (**b**, $X^2_{1,49}$ =4.872, P=0.0273) and greater maximal distance (**c**, $X^2_{1,49}$ =6.467, P=0.011) of dispersal from the initial inoculum, in comparison to double mutant colonies (Δ*geoA* Δ*mibAB;* n=25), in an assay on agar plates. The boxplots show medians, upper and lower quartiles as well as the span of data points that are within 1.5 times the interquartile range.


a
b
e
Springtail captures per trap
Unbaited trap
Culture medium (agar plate)
S. coeficolor
Control
wild-type
ΔgeoA
ns
f
Geosmin
2-MIB
g
Δgeo
ΔgeoA ΔmibAB
Ratio of attraction to odor arms ± C.I.
c
Sticky board
Petri dish with culture
d
F. candida
Odor release
Charcoal filtered air stream
Upwind attraction and odor choice


Volatile sample
of S. coelicolor
FID
GC
EAD
2-Methylisoborneol
OH
Geosmin
OH
FID
0.5 mV/Div
α-Pinene
D-Limonene
2-Methylisoborneol
Geosmin
Germacrene D
Germacradienol
EAD
0.2 mV/Div
Retention time (1 min/Div)


a
wt
bldM
whiA
whiB
whiG
whiH
whiI
log2 Expression
Time of development (h)
b
c
BldM
ChIP-seq
WhiH
ChIP-seq
Normalized IP - Total coverage
Genomic position
0268
geoA
7109
eshA
mibA


a
b
Number of newly formed colonies
80
60
40
20
0
*
ΔgeoA ΔmibAB
wild-type
c
Maximal distance of disperal from initial inoculum (mm)
80
60
40
20
0
*
ΔgeoA ΔmibAB
wild-type