Burkholderia bacteria use chemotaxis to find social amoeba *Dictyostelium discoideum* hosts

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Conflict of interest

The authors declare no conflict of interest.

Running Title

Partner finding in the *Dictyostelium-Burkholderia* symbiosis

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Subject Categories

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Abstract

A key question in cooperation is how to find the right partners and maintain cooperative relationships. This is especially challenging for horizontally transferred bacterial symbionts where relationships must be repeatedly established anew. In the social amoeba *Dictyostelium discoideum* farming symbiosis, two species of inedible *Burkholderia* bacteria (*Burkholderia agricola* and *Burkholderia hayleyi*) initiate stable associations with naive *D. discoideum* hosts and cause carriage of additional bacterial species. However, it is not clear how the association between *D. discoideum* and its carried *Burkholderia* is formed and maintained. Here, we look at precisely how *Burkholderia* finds its hosts. We found that both species of *Burkholderia* clones isolated from *D. discoideum*, but not other tested *Burkholderia* species, are attracted to *D. discoideum* supernatant, showing that the association is not simply the result of haphazard engulfment by the amoebas. The chemotactic responses are affected by both partners. We find evidence that *B. hayleyi* prefers *D. discoideum* clones that currently or previously carried *Burkholderia,* while *B. agricola* does not show this preference. However, we find no evidence of *Burkholderia* preference for their own host clone or for other hosts of their own species. We further investigate the chemical differences of *D. discoideum* supernatants that might explain the patterns shown above using a mass spectrometry based metabolomics approach. These results show that these bacterial symbionts are able to preferentially find and to some extent choose

their unicellular partners. In addition, this study also suggests that bacteria can actively search for and target phagocytic cells, which may help us better understand how bacteria interact with immune systems.

Introduction

Mutualistic cooperation occurs at all levels of life. Cooperators often have mechanisms to find partners and even to choose good partners over bad ones. Finding a partner is an important aspect of mutualistic associations, particularly for the question of specialization. If partner association is haphazard, or if partner finding abilities are weak, selection for mutualism may be weakened or countered by the need to retain adaptations for surviving and reproducing in the absence of partners. When partner finding is reliable, partners can more readily evolve to specialize on each other. Mutualistic cooperation can also be favored by partner choice, which requires partner finding that is effective enough to offer choices (Noë, 2001). However, in mutualisms with vertical transmission of symbionts, partner-finding adaptations may not be necessary and could be lost. At this point, specialization to particular lineages can become extreme.

Partner finding can have different modalities. For instance, in the pollination systems, plants often use visual and/or olfactory signals to attract pollinators (Schaefer *et al.*, 2004; Turchetto *et al.*, 2014). For mate choice in animals, mate finding can involve visual, olfactory and/or auditory signals in different taxa (Candolin, 2003; Andersson and Simmons, 2006). For microbes, which lack a sensory nervous system, partner finding is often mediated through chemicals either at a distance such as quorum sensing in bacteria (Miller and Bassler, 2001), or via contact dependent recognition such as in social amoeba (Strassmann *et al.*, 2011) and myxobacteria (Velicer and Vos, 2009).

When bacterial symbionts actively find partners, it is likely to be through chemotaxis. Chemotaxis is the directed movement of cells in a chemical gradient, which plays a major role in many important biological processes (Wadhams and Armitage, 2004; Bagorda and Parent, 2008). Multicellular organisms need chemotaxis for various developmental functions (Dormann and Weijer, 2003; 2006) (for example, embryology, migration, and aggregation) and it is also involved in processes like cancer metastasis (Roussos *et al.*, 2011). Bacteria use it to search for food, avoid toxins, and respond to changing environments (Wadhams and Armitage, 2004). Bacterial chemotaxis can play important roles in symbiotic associations. Several studies have reported evidence of chemotaxis of bacterial symbionts toward multicellular eukaryotic hosts, including animals (Nyholm and Mcfall-Ngai, 2004a; Garren *et al.*, 2014; Tout *et al.*, 2015), plants (Broek and Vanderleyden, 1995; Sood, 2003; Nilsson *et al.*, 2006; Kiers and Denison, 2008) and fungi (Haq *et al.*, 2014; Stopnisek *et al.*, 2016).

There is little empirical evidence that bacterial chemotaxis is important in symbioses with unicellular protist hosts. Protists are an extremely diverse paraphyletic grouping of generally unicellular eukaryotic organisms that are not animals, plants, or fungi (Adl *et al.*, 2012). They can interact with bacteria in multiple ways. Some of them are predators of bacteria, others are victims of bacterial pathogens, and still others can even form symbiotic interactions (Greub and Raoult, 2004; Brock *et al.*, 2011; Brock *et al.*, 2013; Stallforth *et al.*, 2013; Schulz *et al.*, 2014; Amaro *et al.*, 2015). However, because of the difficulties in cultivation of protists as well as the commonness of unculturable bacterial symbionts, experimental studies of symbiotic relationships between protists and bacteria so far have been limited to a few cultured model systems (Brock *et* *al.*, 2011; Amin *et al.*, 2012; Dziallas *et al.*, 2012; Brock *et al.*, 2013; Stallforth *et al.*, 2013; DiSalvo *et al.*, 2015; Brock *et al.*, 2016; Boscaro *et al.*, 2017). Emerging techniques such as single-cell sequencing could help to discover potential protist-bacteria interactions (Martinez-Garcia *et al.*, 2012). Still, little is known about partner attraction and choice in protist-bacteria interactions. Progress is most likely or be made in simple model systems where the impact of different partners can be understood and manipulated.

The amoeba proto-farming symbiosis with bacteria is a promising system for gaining insight into the question about how cooperation in protist – bacteria interactions forms and is maintained (Brock *et al.*, 2011; Brock *et al.*, 2013; Stallforth *et al.*, 2013; DiSalvo *et al.*, 2014; DiSalvo *et al.*, 2015; Brock *et al.*, 2016). *D. discoideum* is a soil dwelling amoeba that is well known for its unusual life cycle (Kessin, 2001). At the unicellular stage, vegetative cells eat bacteria through phagocytosis and divide through binary fission. When bacterial prey are exhausted, amoebas aggregate to form multicellular migratory slugs which ultimately become fruiting bodies. About 20% of the cells die to form a stalk while the rest differentiate into spores in the sorus, which sits atop the stalk (Kessin, 2001). Some wild amoeba clones were collected in association with different bacterial partners that can potentially be used as food and/or weapons (Brock *et al.*, 2011; Brock *et al.*, 2013; Stallforth *et al.*, 2013). We call these clones farmers because they can seed and defend their crops in new environments (Brock *et al.*, 2011). However, farming status is caused by key bacteria associates belonging to the genus *Burkholderia* (DiSalvo *et al.*, 2015). Two clades (now named as *B. agricola* and *B. hayleyi*, and the amoeba clones that are carrying them are called *B. agricola* farmers and *B. hayleyi* farmers, respectively) of *Burkholderia* can initiate a stable association with naive *D. discoideum* hosts and are found within *Dictyostelium* cells and spores. They are not good food sources for the amoebas but they benefit the amoebas by causing carriage of additional bacterial species some of which are released to seed new food populations. The *Burkholderia* presumably benefit by living inside the amoebas and dispersing in amoeba spores. Curing the amoebas of these *Burkholderia* eliminates this farming trait (DiSalvo *et al.*, 2015).

Both *B. agricola* and *B. hayleyi* can live on their own and can colonize new amoeba clones, indicating they are facultative symbionts, probably with some horizontal transmission. This raises the question that how the association between *D. discoideum* and its carried *Burkholderia* is formed and maintained. It could be accidental, simply happening when the carried *Burkholderia* are randomly ingested by the amoeba host. Alternatively, *Burkholderia* symbionts may be able to preferentially find and choose their social amoeba partners. In this study, we will test these hypotheses. Specifically, we conducted chemotaxis and metabolomics experiments to answer the following questions: 1) Are *Burkholderia* symbionts attracted to their amoeba hosts? 2) How specific are these choices? 3) What small molecules are involved in these choices?

Materials and methods

Wild *D. discoideum* clones, media and culture conditions

This study used wild *D. discoideum* clones (N = 15) collected at Mountain Lake Biological Station in Virginia (clones with a QS designation, 37º21' N, 80º31' W) and Little Butt's Gap, North Carolina (clones with a NC designation, 35°46' N, 82°20' W). We cured farmer *D. discoideum* clones by treating them with tetracycline (DiSalvo *et al.*, 2015). We grew *D. discoideum* from previously frozen spores on SM/5 agar plates (2 g glucose, 2 g BactoPeptone (Oxoid), 2 g yeast extract (Oxoid), 0.2 g $MgCl_2$, 1.9 g KH_2PO_4, 1 g K_2HPO_4 and 15 g agar per liter) with food bacterium *Klebsiella pneumoniae* at room temperature (21℃). Specific clones used in this study are listed in Table S1.

Burkholderia isolates from wild *D. discoideum* farmers

This experiment used carried *Burkholderia* isolates (N = 6) described in previous studies (DiSalvo *et al.*, 2015). We spotted the sorus contents of farmer clones individually on SM/5 agar plates and assessed bacterial growth after 7 days at room temperature. We isolated single colonies from these spots by restreaking bacteria on SM/5 agar medium up to three times. We then identified clones via 16s rRNA gene sequencing (Brock *et al.*, 2011; DiSalvo *et al.*, 2015). Phylogenetic analyses show these carried *Burkholderia* belong to two clades (now named as *B. agricola* and *B. hayleyi*) (DiSalvo *et al.*, 2015). We tested three *B. agricola* (B1qs70, B1qs159 and B1nc21) and three *B. hayleyi* (B2qs11, B2qs21 and B2nc28) *Burkholderia* clones. We also used three non-carried *Burkholderia* species (*Burkholderia unamae*, *Burkholderia tuberum*, *Burkholderia silvatlantica*) to see if attraction to *D. discoideum* is specific to the carried species (Figure 1). Specific isolates used in this study are listed in Table S1. Recently, it has been proposed to split the genus *Burkholderia* into two genera according to phylogenetic clustering: (1) a genus retaining this name (mostly animal and plant pathogens) and (2) the genus *Paraburkholderia* (environmental bacteria) (Sawana *et al.*, 2014). However, there are debates about this reclassification (Vandamme *et al.*, 2017). Therefore, we continue to use the original (*Burkholderia*) names for our species.

Motility test of *Burkholderia* isolates

Motility is the ability to move spontaneously and actively. We tested the motility of carried *Burkholderia* isolates (N = 6) using swim and swarm plate assays according to procedures described earlier (Ha *et al.*, 2014a; b). For the swim assay, we prepared and used the swim plates (SM/5 supplemented with 0.3% agar) the same day. For each isolate, we prepared the bacteria by starting from the frozen clonal isolate and then incubating at room temperature (21℃) until stationary phase was reached. We then inoculated 10 µL of bacteria suspensions (optical density 2.0) at the center of the plates, stabbing the pipette tip into the agar during inoculation (Ha *et al.*, 2014a). For the swarm assay, we also prepared and used the swarm (SM/5 supplemented with 0.8% agar) plates the same day. We inoculated 10 µL of bacteria suspensions (optical density 2.0) at the center of the plates with the pipette tip close to the agar surface during inoculation (Ha *et al.*, 2014b). We inoculated all plates for 48 hours at 30℃ and plates were kept upright and not inverted. At 48 hours we measured the diameter of the colonial growth. We used non-motile (*K. pneumoniae*) and three motile (*Burkholderia unamae*, *Burkholderia tuberum*, *Burkholderia silvatlantica*) controls (Vandamme *et al.*, 2002; Caballero-Mellado *et al.*, 2004; Perin *et al.*, 2006). We did three replicates for each strain.

Preparation of supernatants with potential *D. discoideum* chemoattractants

We prepared *D. discoideum* supernatants (N = 15) from log-growth amoebas for the chemotaxis experiment. We prepared log-growth amoebas by plating 2×10^5 spores in 200 µl *K. pneumoniae* suspension in starvation buffer (2.25 g KH_2PO_4 and 0.67 g K_2HPO_4 per liter H_2O) at an OD of 1.5 on SM/5 plates. We have previously determined that spore germination and amoeba log growth occurs at about 32–36 h after plating (Brock *et al.*, 2013). When clones reached log-phase growth, we collected amoebas using ice-cold starvation buffer and centrifuged the collected amoebas/bacterial suspension at 1,500 g for 3 min to wash the amoebas clean of bacteria. We washed the pelleted amoebas in an excess volume of ice-cold starvation buffer three to four times to remove remaining bacteria.

For the experiment, we placed 10^8 amoebas in a 15-ml conical tube containing starvation buffer and we used a New Brunswick C1 Platform Shaker set at speed 25 to gently rotate the amoeba suspension for 8 h at 21°C. At 8 h, we isolated the supernatant from the amoebas by centrifuging the amoeba suspension in an Eppendorf Centrifuge 5804 R at 1,500 g for 3 min. We further isolated the supernatants in an Eppendorf Centrifuge 5804 R at 12,000 g for 30 min at 4°C. We decanted the supernatant to a fresh, sterile conical tube and placed the tube at 4 °C. We then filtered the supernatants through a 0.2-mm sterile syringe filter (Millipore) and kept them at 4°C until experimental set-up. To test if those amoebas were still viable after 8 h, we plated the amoebas on SM/5 plate to see if they can aggregate and form fruiting bodies. All tested *D. discoideum* clones were still able to form fruiting bodies.

Capillary chemotaxis assay for carried *Burkholderia*

To investigate the chemotactic responses of carried *Burkholderia* (N = 6) in response to amoeba supernatants (N = 15), we used a capillary assay described previously (Mazumder *et al.*, 1999). We used an Eppendorf tube as a chamber for holding 200 µl of bacterial suspension (OD 2.5) in starvation buffer. We used a 2-cm 25-gauge needle (Becton Dickinson) as the chemotaxis capillary and attached it to a 1-mL tuberculin syringe (Becton Dickinson) containing a 100 µl of amoeba supernatant. After 4 h incubation at room temperature, we removed the needle syringe from the bacterial suspension and collected the contents. We measured the accumulation of bacteria in the capillaries using flow cytometry. We diluted the bacterial contents to a final volume of 1.0 mL. For each assay, we added 1 µL of SYTO® BC bacteria stain (Component A) and 10 µL of microsphere suspension (Component B) into the diluted bacteria. The mixture was incubated at room temperature for 10 minutes. We then assayed the colored bacteria in a flow cytometer equipped with a laser emitting at 488 nm. Fluorescence is collected in the fluorescein channel. The forward scatter and fluorescence were collected with logarithmic signal amplification. We calculated the number of bacteria using the number of microspheres (10^6 per mL) as a standard. There are three biological replicates for each experimental unit and each replicate was measured three times. This design resulted in a total of 6 (carried *Burkholderia*) × 16 (amoeba supernatants and blank control) × 3 (biological replicates) × 3 (technical replicates for bacterial counting) = 864 experimental units for the chemotaxis assay. This means there are 96 host-*Burkholderia* combinations, and for each combination there are 3 biological and 3 technical replicates. We used the mean of each host-*Burkholderia* combination for further analysis.

Host preferences of *Burkholderia*

Next, we want to know how specific these chemotactic responses are and whether farmer associated *Burkholderia* would prefer their original farmer hosts. We investigated host preferences at both species and clone levels. We used the same chemotaxis dataset (N=96) for this purpose. However, we analyzed and plotted *B. agricola* and *B. hayleyi* separately.

To investigate if carried *Burkholderia* are more attracted to host supernatants than non-carried *Burkholderia*, we used 3 non-carried *Burkholderia* species (*B. unamae*, *B. tuberum*, *and B. silvatlantica*) to see if attraction to *D. discoideum* is specific to the carried species. We used the same capillary assay describes above.

Burkholderia – Burkholderia attractions

To investigate the potential chemotactic responses of carried *Burkholderia* (N = 6) in response to their own secretions (N = 6), we used the same capillary assay described above, except that amoeba supernatants were replaced by *Burkholderia* supernatants. To prepare *Burkholderia* supernatants, we placed 8 ml of bacterial suspension (OD 1.5, 10^9 cells) in a 15-ml conical tube containing starvation buffer and we used a New Brunswick C1 Platform Shaker set at speed 25 to gently rotate the *Burkholderia* suspension for 8 h at 21℃.We isolated the supernatants in an Eppendorf Centrifuge 5804 R at 12,000 g for 30 min at 4℃. We then filtered the supernatants through a 0.2-mm sterile syringe filter (Millipore) and use them for a modified capillary assay (see above session). This design resulted in a total of 6 (*Burkholderia*) × 7 (*Burkholderia* supernatants and blank) × 3 (replicates) = 126 experimental units.

Identification of potential attracting molecules using LC-MS/MS

We identified the potential attracting molecules in all 15 amoeba supernatants using liquid chromatography-tandem mass spectrometry (LC-MS/MS). We performed LC-MS/MS experiments on the Thermo Dionex UltiMate 3000 HPLC (Waltham, MA) and the Thermo Q Exactive Plus mass spectrometer (Waltham, MA). We centrifuged the samples at 8000 rcf for 3 min prior to analysis. We loaded 5 μL from each sample to the Thermo Acclaim Pepmap 100 C18 column (Waltham, MA) by solvent A at 5 μL/min flow rate. After that, samples were eluted by a linear gradient at 500 nL/min and further separated by a homemade column. The column was 100-μm-inner-diameter and 12-cm-length silica tubing packed with Magic C18 AQ reversed phase material. The gradient was increased from 98% solvent A and 2% solvent B to 2% A and 98% B in 50 min. Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in 80% acetonitrile and 20% water (v:v). The analyte was ionized and introduced into the mass spectrometer by the Thermo Nanospray Flex Ion Source (Waltham, MA) in positive mode. Each amoeba supernatant was run once due to logistic reason, resulting a total of 15 datasets for 15 amoeba supernatants.

Metabolomics analysis

Data from LC-MS/MS based untargeted metabolomic experiments are highly complex, therefore we subjected the results to analysis by XCMS Online (Smith *et al.*, 2006; Tautenhahn *et al.*, 2012; Gowda *et al.*, 2014). The data files were converted to mzXML format by MSConvert with peak picking filter enabled. The parameters were as follows: centWave for feature detection, Δ m/z = 5 ppm, min peak width = 5 s, max peak width = 40s; obiwarp for retention time correction with profStep = 1; minfrac = 0.5, bw = 10, and mzwid = 0.025 for chromatographic alignment.

Compounds of interest can sometimes be identified from the differences between two groups (Gowda *et al.*, 2014). Because the strongest group differences from our capillary assays was between supernatants from *D. discoideum* (farmers) carrying *Burkholderia* versus *D. discoideum* not carrying *Burkholderia* (non-farmers and cured farmers), we performed a two-group comparison between *D. discoideum* carrying (six farmers) and not carrying (six cured farmers and three non-farmers) *Burkholderia* by processing pairwise jobs at XCMS Online (Gowda *et al.*, 2014). We selected Mann-Whitney test (with false discovery rate adjusted p-value) for the two-group comparisons at XCMS Online (Gowda *et al.*, 2014). For the features that are not identified by MS/MS, we identified them by searching their accurate mass in the METLIN database. All candidates have a mass differences less than 5ppm, which is limited by the resolution of the mass spectrometer. Adducts such as $[M+NH_4]^+$ are included, but restricted to the specified charge states of the features. For unknown charge states, both +1 and +2 charges are included.

Statistical analyses

Partner attraction and choice

For chemotaxis experiments, the accumulation of *Burkholderia* in response to the amoebal secretions was expressed in terms of a chemotaxis index. Because tested *Burkholderia* differ somewhat in their motility (Figure S1), we normalized the chemotaxis index using the motility data. We calculated the chemotaxis index value as: number of bacteria/motility (diameter of swimming assay). We performed the experiment as a nested factorial design.

We analyzed the chemotaxis index data with a generalized linear model (GLM) with lognormal distribution in the Genmod procedure of SAS 9.3 (SAS Institute, Inc.). All blank controls had zero bacteria, so to simplify the model and eliminate this source of non-normality, we excluded them from the model. In these analyses, symbiont species (two levels: *B. agricola* and *B. hayleyi*), Host type (five levels: non-farmers, *B. agricola* farmers, *B. hayleyi* farmers, cured *B. agricola* farmers and cured *B. hayleyi* farmers) and their interactions were used as fixed factors. *Burkholderia* clone and amoeba clone were nested within symbiont species and host type, respectively. Relevant pairwise comparisons were conducted using Tukey's post hoc tests.

Because we only had three levels of *Burkholderia* clones and host clones, we didn’t assign them as random factors in the model (Bolker *et al.*, 2009). However, we did run a separate generalized linear mixed model (in the Glimmix procedure of SAS 9.3 (SAS Institute, Inc.)) in which they were included as random factors (nested within symbiont species and host type), and the results were consistent with our fixed-effects model (Table S2).

A significant symbiont species main effect would indicate chemotactic divergence between the two *Burkholderia* species, a significant host type main effect would indicate that different host types invoke different chemotactic responses, and a significant symbiont species × host type interaction would indicate that chemotactic responses are affected by both partners.

Host preferences of Burkholderia

To test host preferences of carried *Burkholderia* at both species and clone levels, we used the same chemotaxis dataset (N=90) for this purpose. However, we analyzed and plotted *B. agricola* and *B. hayleyi* separately. Host preference by carried *Burkholderia* at species level (N = 90) was analyzed using One-way nested ANOVA, in which *Burkholderia* clone was nested within source of supernatant (other species, own species and own species cured). Relevant pairwise comparisons were conducted using Tukey's post hoc tests. Host preference by *Burkholderia* at clone level (N = 90) was analyzed using One-way nested ANOVA, in which *Burkholderia* clone was nested within source of supernatant (other host, own host and own host cured). Relevant pairwise comparisons were conducted using Tukey's post hoc tests.

To investigate host attraction of carried versus non-carried *Burkholderia*, we used the same chemotaxis dataset (N=90), as well as an additional dataset from non-carried *Burkholderia* (N=27). We compared the chemotaxis index among *B. agricola*, *B. hayleyi* and non-carried *Burkholderia* using One-way nested ANOVA, in which *Burkholderia* clone was nested within symbiont type (*B. agricola*, *B. hayleyi* and non-carried *Burkholderia*). Relevant pairwise comparisons were conducted using Tukey's post hoc tests.

Chemotaxis index data were log-transformed to improve normality. Because all blank controls had zero bacteria, they were excluded from the analyses. Transformed data passed the normality test (Kolmogorov–Smirnov test) and tested for homogeneity of variance (Levene’s test).

Results

***Burkholderia* motility**

All Burkholderia in symbiosis with D. discoideum are motile but do not have social swarming motility

Chemoattraction would have little effect unless the carried *Burkholderia* are motile, so first of all we want to know if they can move. We tested this by using standard plate-based bacterial swimming and swarming assays (Ha *et al.*, 2014a; b), with non-motile *Klebsiella pneumoniae* as a negative control and three motile *Burkholderia* (*B. unamae* (Caballero-Mellado *et al.*, 2004), *B. tuberum* (Vandamme *et al.*, 2002) and *B. silvatlantica* (Perin *et al.*, 2006)) as positive controls. We found that all six carried *Burkholderia* clones are motile, though they differ in motility (movement diameters across the plate ranging from 2.078 to 2.951 cm while non-motile *K. pneumoniae* is 0.853 cm, Figure S1). However, there are no significant differences among *B. agricola* (B1qs70, B1qs159 and B1nc21), *B. hayleyi* (B2qs11, B2qs21 and B2nc28), and the three non-carried *Burkholderia* species (N = 9, $F_{2,6} = 0.313$, $P = 0.743$), indicating that this is likely to be an ancestral trait not particularly evolved for symbiosis. In the swarm assay, all nine tested clones lack swarming ability (Figure S2). So *Burkholderia* swim but do not do so socially.

Partner attraction and choice

Carried Burkholderia chemotax towards amoeba supernatant and B. hayleyi prefers farmer supernatant

To test whether carried *Burkholderia* are attracted to their amoeba hosts, we used amoeba supernatant as a stand-in for the actual amoebas to rule out any amoeba action. We used the supernatants as chemoattractants in a capillary chemotaxis assay. We tested three clones each of *B. agricola* and *B. hayleyi* with supernatants from five host types: *B. agricola* farmers, *B. hayleyi* farmers, non-farmers, cured *B. agricola* farmers and cured *B. hayleyi* farmers, using three different amoeba clones from each. Amoeba supernatants invoked a strong chemotactic response by the carried *Burkholderia* (Figure 2), as shown by the accumulation of bacteria in supernatant-containing syringes while no bacteria were found in the control syringes (containing KK2 buffer). To further investigate what kind of partner attraction exists, we analyzed the dataset using a generalized linear model.

We found a significant symbiont species × host type interaction (Table 1A), indicating two *Burkholderia* species behave differently in response to the five different host types (Figure 2). To establish the nature of these interactions, we next investigated the potential effects of the symbionts and hosts in chemotactic responses by host type (Table 1B) and by symbiont species (Table 1C) respectively.

When analyzed by host type, pairwise comparisons suggest that there was no difference between *B. agricola* and *B. hayleyi* in response to all host types (Table 1B, Figure 2). These results suggest that both *B. agricola* and *B. hayleyi* are equally attracted to amoeba supernatants.

When analyzed by symbiont species, multiple comparisons suggest that different host types invoke different chemotactic responses (Table 1C, Figure 2). All host types induced equal responses to *B. agricola* (Table 1C, Figure 2). However, *B. agricola* farmers and *B. hayleyi* farmers are generally more attractive to *B. hayleyi* compared to non-farmers (Table 1C, Figure 2).

Removal of Burkholderia from farmer clones decreases chemotactic responses in B. hayleyi

To investigate if the presence of *Burkholderia* would make any differences to the chemotactic responses, we removed the *Burkholderia* from both *B. agricola* and *B. hayleyi* farmers. When *Burkholderia* were removed from their farmer hosts, these cured farmers induced similar chemotactic responses as non-farmers (Figure 2, Table 1C). *B. agricola* and *B. hayleyi* were equally attracted to non-farmers and cured farmers (Figure 2, Table 1C). In addition, two species respond differently to curing of hosts (Figure 2, Table 1C). Removal of *B. agricola* seemed to have no effect to their chemotactic responses (Figure 2, Table 1C). However, removal of *B. hayleyi* decreases the chemotactic responses compared to uncured farmers (Figure 2, Table 1C, both *B. agricola* and *B. hayleyi* farmers).

Taken together, these results suggest that all carried *Burkholderia* are attracted to *D. discoideum* clones from which they were isolated. However, *B. hayleyi* exhibits specific choice towards the uncured farmer *D. discoideum* clones while *B. agricola* does not.

Host preferences of ***Burkholderia***

Next, we want to know how specific these chemotactic responses are and whether farmer associated *Burkholderia* would prefer their original farmer hosts. We investigated host preferences at three different levels and ask three corresponding questions: 1) Are carried *Burkholderia* more attracted to host secretions than non-carried *Burkholderia*? 2) Are *B. agricola* and *B. hayleyi Burkholderia* more attracted to their own hosts of their species; 3) Is each *Burkholderia* clone more attracted to its own host?

Host attraction of carried Burkholderia versus non-carried bacteria

We find strong evidence that chemotactic responses to *D. discoideum* hosts are relatively specific to carried *Burkholderia*. There are significant differences in attraction to *D. discoideum* among *B. agricola*, *B. hayleyi* and the three non-carried *Burkholderia* tested (One way nested ANOVA, $F_{2, 6} = 29.393$, $P = 0.001$). Both *B. agricola* (pairwise Tukey tests, $P < 0.001$) and *B. hayleyi* (pairwise Tukey tests, $P < 0.001$) are more attracted to host secretions than are non-carried

Burkholderia (Figure 3A). All blank controls invoked zero response (no bacteria in both blank controls, Figure 3A). *B. tuberum*, which is within the same major clade as the carried *Burkholderia* (Estrada-de los Santos *et al.*, 2013; DiSalvo *et al.*, 2015), does not show higher attraction to *D. discoideum* compared to non-carried *B. unamae* (Tukey post hoc test, $P = 0.198$, Figure S3) and *B. silvatlantica* (Tukey post hoc test, $P = 0.677$, Figure S3).

To further investigate how other bacteria respond to amoeba secretions, we performed additional experiments on 9 strains of 7 different bacterial species (*Pseudomonas aeruginosa*, *Serratia marcescens*, *Bacillus subtilus*, *Staphylococcus aureus*, *Burkholderia fungorum*, *Burkholderia xenovorans*, *Escherichia coli (536)*, *E. coli (ColF6c)*, *E. coli (IA152)*). For logistical reasons, we only tested them on one amoeba supernatant (QS9). We found that all tested bacterial species showed some degree of attraction to amoebal secretion (Figure S4), and they differed in their chemotactic responses (one way ANOVA, $P < 0.001$, Figure S4). However, these attractions are significantly weaker compared to those of carried symbionts (Figure S5). Carried symbionts showed significantly higher attraction than non-carried *Burkholderia* (pairwise Tukey tests, $P = 0.018$, Figure S5) and other bacterial species (pairwise Tukey tests, $P <0.001$, Figure S5) to amoeba secretion, while there is no difference between non-carried *Burkholderia* and other bacterial species (pairwise Tukey tests, $P = 0.277$ Figure S5).

Preference for hosts of the same Burkholderia species

We find no evidence that *B. agricola* and *B. hayleyi* are more attracted to hosts of their own species (Figure 3B). For *B. agricola*, the one-way ANOVA of chemotaxis index on the measure of host preference revealed no statistically significant main effect (One way nested ANOVA, $F_{2, 6} = 0.690$, $P = 0.523$), indicating that no host preference was found in *B. agricola* for *B. agricola* hosts (Figure 3B). For *B. hayleyi*, no statistically significant main effect was found either (One way nested ANOVA, $F_{2,6} = 1.197$ $P = 0.354$).

Host preference of specific clone

At the clone level, the statistical patterns here are exactly the same as in the previous section: there is no evidence that shows *Burkholderia* clones are more attracted to their own host (Figure 3C). Again, *B. agricola* show no host specificity (one way nested ANOVA, $F_{2,6} = 0.182$, $P = 0.835$, Figure 3C). *B. hayleyi* also have no statistically significant main effect (One way nested ANOVA, $F_{2,6} = 0.897$ $P = 0.430$).

Contribution of *Burkholderia – Burkholderia* attractions to overall chemotactic responses

As farmer clones are carrying *Burkholderia* throughout our experimental procedure (except in the cured treatments), it is possible that these carried *Burkholderia* may also secrete attractive compounds into the amoeba supernatants being tested. Therefore, our overall chemotactic responses could be affected by the *Burkholderia – Burkholderia* attractions. To test this, we performed an additional experiment to test the attractiveness of *Burkholderia* secretions. We prepared *Burkholderia* supernatants from equal (or slightly higher) number of *Burkholderia* (10^9 cells) as in the amoeba supernatant experiment (versus 10^8 amoeba cells, and each amoeba has 2 – 10 *Burkholderia* bacteria inside, L. Shu, personal observations).

Overall, *Burkholderia* supernatants invoked a very minor chemotactic response (chemotaxis index: 235.51 ± 43.98, mean ± S.E.), which is less than 1% compared to that of amoeba supernatants (chemotaxis index: 40247.31 ± 1709.207, mean ± S.E.) by the carried *Burkholderia* (Figure 2 and Figure 4). A Mann-Whitney U test indicated that amoeba supernatants were statistically significantly more attractive to carried *Burkholderia* than the *Burkholderia* supernatants ($N_{amoeba} = 6$, $N_{Burkholderia} = 6$, U = 0.000, *P* = 0.002). Taken together, these data suggest that the global chemotactic responses are unlikely to be strongly affected by the *Burkholderia – Burkholderia* attractions.

Within the *Burkholderia – Burkholderia* attraction experiment, most *Burkholderia* supernatants actually invoked zero chemotactic response (Figure 4). Interestingly, most of the chemotaxis response happened in one specific *B. agricola* clone, B1qs70 (Figure 4). Clone B1qs70 both attracted most other clones and was attracted to most other clones. However, it is not clear why this clone shows a different pattern.

Composition of the attractive supernatant

Global analysis of extracellular metabolites

To begin to identify potential chemoattractants and to look for chemical differences that might explain the patterns shown above, we explored the extracellular metabolites of each *D. discoideum* clone using an untargeted metabolomics approach. We found both qualitative and quantitative variations in base peak intensity of chromatograms across different *D. discoideum* clones (Figure 5A).

To investigate the global pattern of *D. discoideum* supernatants, we further identified the metabolites using HPLC-MS/MS and analyzed the dataset using XCMS Online (Tautenhahn *et al.*, 2012; Gowda *et al.*, 2014). XCMS is a widely used, cloud-based platform designed to process untargeted metabolomics data (Smith *et al.*, 2006; Tautenhahn *et al.*, 2012; Huan *et al.*, 2017). Overall, a large number of features was identified in each clone, ranging from 16214 to 22431 (Figure 5B). To visualize the level of similarity of individual *D. discoideum* clones, we analyzed the dataset using a non-metric multidimensional scaling (Figure 5C). Farmers (blue dots) and non-farmers (black dots) are generally clustered together. However, cured farmers (red dots) are scattered and distinct, with one exception, from both farmers and non-farmers (Figure 5C). Interestingly, based on the chemotaxis experiment, non-farmers and cured farmers are equally attractive to both species of *Burkholderia*, even though they actually differ widely in their metabolomics compositions. In addition, we found considerable variation within cured farmers. These results suggest that cured farmers are not as similar to non-farmers as we expected, and the presence of *Burkholderia* makes a big difference to the metabolomics profiles of *D. discoideum* hosts.

Identification of potential chemoattractants

Compounds of interest can sometimes be identified from the differences between two groups (Gowda *et al.*, 2014). The strongest group difference from our capillary assays was between supernatants from *D. discoideum* (farmers) carrying *Burkholderia* versus *D. discoideum* not carrying *Burkholderia* (non-farmers and cured farmers). This approach might identify compounds that were specific to farmers or it might identify compounds that were generally important in attracting *Burkholderia*, but present more in farmers.

We therefore performed a group comparison of metabolites between farmers and non-farmers/cured farmers. We identified 155 features that were altered with fold changes greater than 2 and p-values less than 0.01 (Figure 6A). Of these, 52 features have higher abundances in farmer groups (Figure 5), so these are candidate chemoattractants to *B. hayleyi*.

One potential caveat of the XCMS approach is its false positive features. To investigate this we validated these 52 features by manually checking the extracted ion chromatogram at XCMS Online and removing the ones with low signal to noise ratio. Furthermore, we found that the isotopic peaks of the same compound could be identified as individual features. Therefore, we checked the isotopic pattern of each feature on their mass spectra in raw data and reassigned the monoisotopic peak as the m/z value. We finally identified 18 features that are candidates of attractants to *B. hayleyi* (Table 2).

Among those 18 features, 12 were isolated by the mass spectrometer to generate fragment ion spectra. MS/MS data of the other 6 features were not obtained due to their low abundance and the limited capability of the mass spectrometer. We tried to identify those 12 features by carefully examining their fragment ion spectra. First, we compared the m/z difference between major peaks against residue mass of the 20 amino acids to distinguish peptides from other metabolites. Incomplete peptide sequences are proposed by following series of b and y ions on the spectra. Because we can't distinguish between b and y ions, the reverse of the sequences shown in Table 2 may also be possible. Interestingly, all 6 peptides we found are proline-rich peptides. Two sets of peptides, m/z = 657.6403 and 663.3154, m/z = 670.2897 and 675.2771, have almost identical fragment ions. They could be peptides with the same sequence but different modifications or adducts. All peptide sequences were blasted against non-redundant protein sequences for *D. discoideum* and *Burkholderia* using BLASTP (Altschul *et al.*, 1997). All peptides yielded perfect hits in the *D. discoideum* database, but none of them did in *Burkholderia*, indicating these peptides are from amoebas rather than *Burkholderia*. Top hits are selected and shown in Table 2.

After identification of peptides, we submitted the MS/MS results of the remaining 6 metabolites to MassBank (Horai *et al.*, 2010), mzCloud (mzcloud.org), HMDB (Wishart *et al.*, 2013) and METLIN (Smith *et al.*, 2005) for spectra matching. However, we were unable to match fragment ion spectra of most of the metabolites. They could be new metabolites or ones in the database without MS/MS information. Their exact mass and assigning MS/MS peaks are listed in Table 2. We identified one feature (m/z = 251.1633) as 3,5-di-tert-butyl-4-hydroxybenzoic acid with high confidence. 3,5-di-tert-butyl-4-hydroxybenzoic acid ($C_{15}H_{22}O_3$) is a carboxylic acid. We are not able to find any related literatures on its biological function. However, some evidence links it to another chemical butylated hydroxytoluene (BHT), which is an artificial antioxidant agent.

For the features that are not identified by MS/MS, we found 2 - 158 candidates for each feature by searching their accurate mass in the METLIN database (Supplementary Information 2). All candidates have a mass difference less than 5ppm, which is limited by the resolution of the mass spectrometer. Due to structure variation and limitation of the database, we are unable to determine the exact structures of those features. However, the compound has an m/z=168.5831 is likely to be a tripeptide and the compound of 496.3019 can be a lipid molecule.

Discussion

This study has identified mechanisms for forming and maintaining *Dictyostelium – Burkholderia* symbioses. We show that partner finding plays a significant role in this mutualistic associations of microbial systems. Out study suggests that active partner finding by bacteria is an important feature not just in mutualisms with animals and plants, but also with protists. Our study also highlights that chemotaxis is an important modality of partner finding, just like visual, olfactory and auditory cues are in other cooperative interactions such as mating choice or pollination.

We can now reject the hypothesis that initiation of associations is no more complex than the haphazard ingestion of *Burkholderia* by amoebas. We can also reject the hypothesis that the attraction to *Dictyostelium* is a trait of *Burkholderia* in general. The genus *Burkholderia* belongs to the class β-Proteobacteria and is widely distributed in the environment. They are abundant in soil and they can be associated with a wide range of plants (Elliott *et al.*, 2009), invertebrates (Kikuchi *et al.*, 2005; Garcia *et al.*, 2014) and fungi (Stopnisek *et al.*, 2016). The two species of carried *Burkholderia* are more attracted to amoeba secretions than are the non-carried *Burkholderia* species we tested. This shows that the association of these two *Burkholderia* species with *D. discoideum* is not a random or accidental infection process. Instead, the association is sought out specifically by those *Burkholderia* that live inside *D. discodeum*, strongly suggesting that this chemotaxis is a partner-finding adaptation. Other non-carried bacterial species did show a very small amount of attraction to *Dictyostelium* supernatants, suggesting that the partner-finding adaptation is likely evolved from a more generalized response.

However, we did not find much evidence for carried *Burkholderia* making specialized choices among hosts. One exception is that the *B. hayleyi*, but not the *B. agricola*, prefers farmer clones over non-farmer and cured farmer clones. Why they would be attracted specifically to infected hosts is uncertain but this evidence might suggest that *B. hayleyi* is more co-evolved with amoeba farmers perhaps because of a longer history of co-evolution with *D. discoideum* than *B. agricola* in nature. To validate this hypothesis, further comparative studies between these two species are needed. However, co-evolution with respect to attraction does not appear to be very specific. *Burkholderia* clones are not specifically attracted to their own particular host clone, nor to other hosts of their own species. This suggests that host clone switching or horizontal transmission may occur often enough to prevent the more specific co-evolution that can occur under strict vertical transmission. Though these bacteria are endosymbionts that live inside the host (DiSalvo *et al.*, 2015), they are apparently not obligate endosymbionts. They can still grow independently of their hosts in the lab, and their retention of motility suggests that they also do so in nature.

This kind of chemotaxis mediated partner finding mechanism has only been reported in the interactions between bacteria and their multicellular eukaryotic hosts (Sood, 2003; Nyholm and McFall-Ngai, 2004b; Butler and Camilli, 2005; Nilsson *et al.*, 2006; Haq *et al.*, 2014; Tout *et al.*, 2015; Stopnisek *et al.*, 2016). For instance, in the rhizobia-legume system free-living rhizobia are attracted to the environment around legume root hairs through chemotaxis (Pandya *et al.*, 1999; Wadhams and Armitage, 2004; Kiers and Denison, 2008). In another model symbiosis, the squid-vibrio system, the marine bacterium *V. fischeri* uses chemotaxis to move towards and colonize the squid (*E. scolopes*) light organs (Nyholm and McFall-Ngai, 2004b). Our study shows that bacteria can use the same partner finding mechanism to interact with unicellular eukaryotic hosts and suggests that bacterial chemotaxis is a general feature of eukaryotic host-bacterial symbioses.

We used metabolomics to try to identify candidate chemoattractant compounds, exploiting differences between farmers versus non-farmers and cured farmers. For our short list of candidates that passed quality controls, six did not yield useful MS/MS data. Among the remaining twelve were six proline-rich peptides. The rest failed to generate hits in the databases, with the exception of 3,5-di-tert-butyl-4-hydroxybenzoic acid. Previous studies suggest that 3,5-di-tert-butyl-4-hydroxybenzoic acid is involved in the metabolic pathway of (BHT) in rat and human (Daniel *et al.*, 1968; Yamamoto *et al.*, 1991). In addition, some phytoplankton, including the green algae *Botryococcus braunii* and three different cyanobacteria (*Cylindrospermopsis raciborskii, Microcystis aeruginosa and Oscillatoria sp.*) are capable of producing BHT, which may link to the reactive oxygen species (ROS) production in these species (Babu and Wu, 2008;

Dey *et al.*, 2016). However, this compound seems unlikely to be of biological origin and could simply be a breakdown product of the preservative BHT (Jon Clardy, pres. comm.). Among the rest of unknown features, additional identification by searching their accurate mass in the METLIN database suggests that the compound has an m/z=168.5831 is likely to be a tripeptide and the compound of 496.3019 can be a lipid molecule (Supplementary Information 2). These results indicate that attraction of the symbionts is likely related to various metabolism processes. However, finding the exact chemoattractants is going to require additional work. Metabolites from amoebae are produced in extremely low quantities, which makes it virtually impossible to do a full structure elucidation via NMR (Barnett and Stallforth, 2017). Due to these difficulties, it is likely that natural products from the social amoebae have to be discovered through heterologous expression of the respective gene clusters (Barnett and Stallforth, 2017).

Our study also has some implications for human disease and health. Amoebas have served as model systems for studying ecology, evolution and biology of pathogenic bacteria (Horn and Wagner, 2004; Hoffmann *et al.*, 2014; Tosetti *et al.*, 2014). *D. discoideum* cells are professional phagocytes, and the core function of the innate immune response is evolutionarily conserved between amoebas and human professional phagocytic cells (Kessin, 2001). Our study shows that bacteria can actively move towards phagocytic cells. This is quite interesting because generally bacteria are thought to evade phagocytic killing rather than running into it (Casadevall, 2008). However, given that amoebas have interacted with bacteria for a long time, even before animals appeared (McFall-Ngai *et al.*, 2013; Strassmann and Shu, 2017), it is not surprising that some bacteria have evolved mechanisms that specifically target and exploit phagocytic cells and this could affect how these bacteria interact with our immune systems.

Future research should address the reverse question, whether *Dictyostelium* is particularly attracted to *B. hayleyi* and *B. agricola*. The fitness consequences to the host are context dependent and sometimes negative; it is costly to carry *Burkholderia* in circumstances (high food) where the farming trait is unnecessary (Brock *et al.*, 2011). If the amoebas are attracted by these *Burkholderia* it would suggest that the relationship is a net beneficial one; if they are repelled, it would suggest the opposite. Future research should also address the genetic basis of these interactions, given that several chemotaxis related genes have been identified in related species (Kim *et al.*, 2007; Kumar *et al.*, 2013; Angus *et al.*, 2014).

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Competing interests

The authors declare no competing interests.

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Shu *et al.* Figure legends

Figure 1. Schematic flow chart of experimental design. The experiment explores if different groups of *Burkholderia* are able to preferentially find and choose their wild *D. discoideum* partners with a chemotaxis assay. The *Burkholderia* phylogeny shows two distinct species of symbionts, *B. hayleyi* and *B. agricola* (DiSalvo *et al.*, 2015), with the position of *B. tuberum* strongly supported by subsequent analyses (T. Hasselkorn, pers. comm.). We used amoeba supernatants as chemoattractants and counted the number of bacteria in the syringe using flow cytometry. We further analyzed the amoeba supernatants using a metabolomics approach. We identified the potential attracting molecules using liquid chromatography/mass spectrometry (LC-MS/MS).

Figure 2. Both species of farmer-associated *Burkholderia* isolates are attracted to amoeba secretions. The figure represents combined boxplot/violin plot diagram. Because all blank controls are zero, they are excluded from the figure (violin plot cannot be created). Results show *D. discoideum* supernatants invoked a strong chemotactic response by the carried *Burkholderia*, and that chemotaxis responses are affected by both partners. Significant differences in chemotactic responses are indicated by different letters, which reflect results of a post hoc Tukey's HSD test (Table 1). Letters apply both within and between panels (letters are the same). However, comparison between the two panels is in a pairwise pattern (vertical columns).

Figure 3. Carried *Burkholderia* are more attracted to *D. discoideum* secretions than are non-carried *Burkholderia*, but they do not prefer hosts of their own clones or species**.** To investigate how specific these chemotactic responses are and whether carried *Burkholderia*

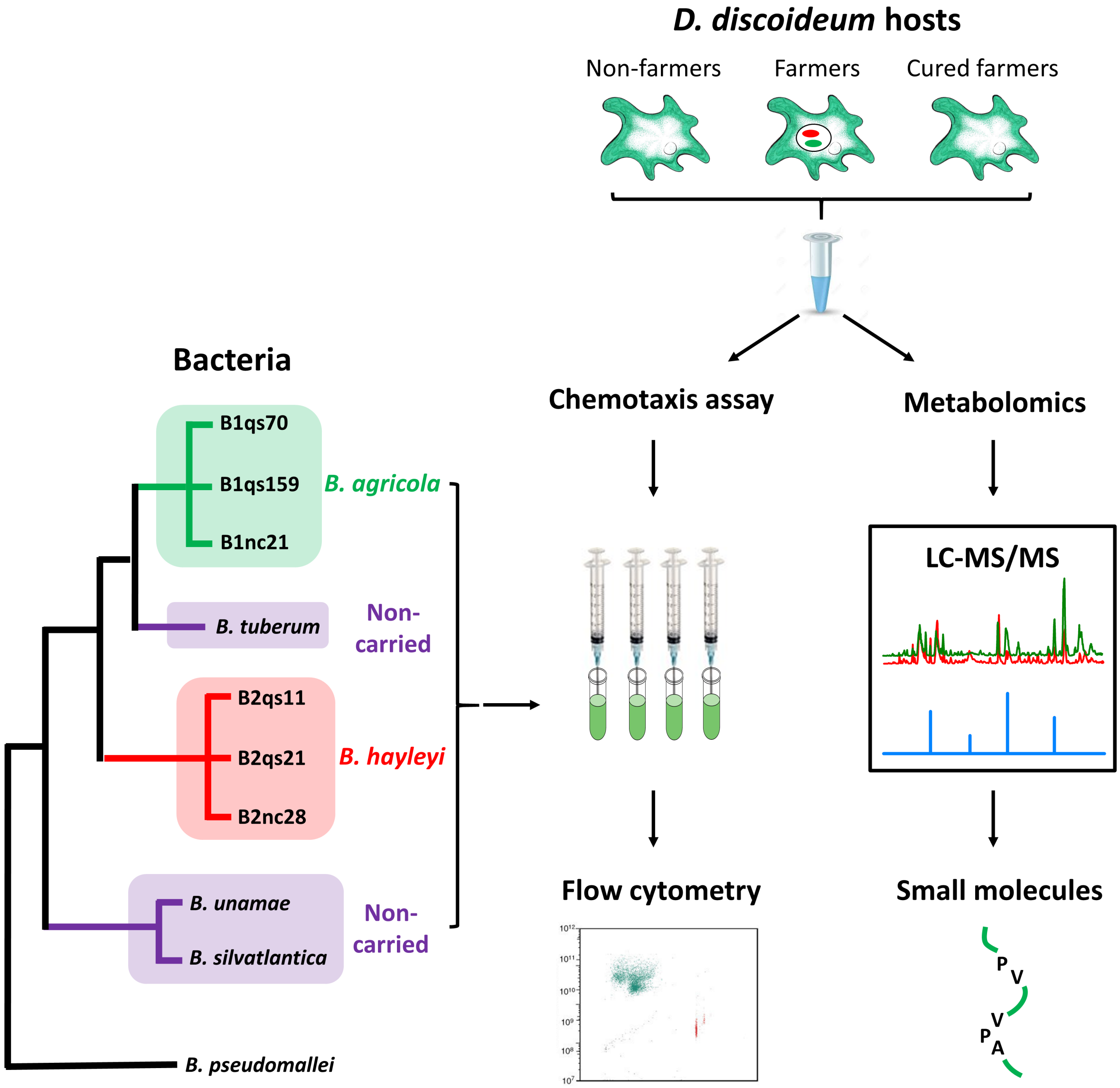
would prefer their original hosts, we studied host preferences at three different levels. The figure shows mean ± SEs of chemotactic responses. A) Carried *Burkholderia* were more attracted to *D. discoideum* secretions (from all clones) than non-carried *Burkholderia*. B) *B. hayleyi* and *B. agricola* did not prefer hosts of their own species. C) Each *Burkholderia* clone did not prefer its own host clone. Significant differences in chemotactic responses are indicated by different letters, which represent results of one-way nested ANOVA test and followed by a post hoc Tukey's honestly significantly different (HSD) test. Letters apply within panel A, B and C. For panel B and C, letters apply within *B. hayleyi* and *B. agricola*.

Figure 4. Only clone *B. agricola* B1qs70 shows much *Burkholderia- Burkholderia.* The figure represents a heat map of the chemotactic responses using *Burkholderia* supernatant or a blank control. The chemotactic responses of the carried *Burkholderia* are indicated by varying color intensities according to the legend at the top of the figure. The Y-axis represents *Burkholderia* supernatants and the X-axis represents each *Burkholderia* clone. The color key represents the value of the chemotaxis index.

Figure 5. Metabolomics analysis of extracellular metabolites in supernatant of *D. discoideum*. Tested clones are identified by QS or NC numbers, with a trailing "C" indicating that the clone was cured of its symbiont. A) HPLC-MS analysis of *D. discoideum* extracellular metabolites. Base peak ion (BPI) chromatograms obtained from the 15 *D. discoideum* clones are shown. The Y-axis represents relative abundance and the X-axis represents retention time. B) Heat map of all identified extracellular metabolites from 15 *D. discoideum* clones. The abundance of each metabolite is indicated by varying color intensities according to the legend at the top of the figure. The color key represents the Z score, the deviation from the mean by standard deviation units. C) Non-Metric multidimensional scaling analysis to show the similarity

of extracellular metabolites from 15 *D. discoideum* clones. Colors indicate different *D. discoideum.* Blue, farmer clones. Black, non-farmer clones. Red, cured farmer clones.

Figure 6. Group comparison to identify farmer specific molecules. Cloud plot of differences between farmers and non-farmers plus cured farmer of 155 features with p-value ≤ 0.01 and fold change ≥ 2. The y coordinate of each feature corresponds to the mass-to-charge ratio of the compound as determined by mass spectrometry. Features whose intensity is increased in farmers are shown on the top plot in green, whereas features whose intensity is decreased in farmers are shown on the bottom plot in Red. The diameter of each bubble corresponds to the log fold change of the feature: the larger the bubble, the larger the fold change. The intensity of the feature's color represents the statistical significance of the fold change, where features with low p-values are brighter compared to features with high p-values.


D. discoideum hosts
Non-farmers
Farmers
Cured farmers
Bacteria
B1qs70
B1qs159
B1nc21
B. agricola
B. tuberum
Non-carried
B2qs11
B2qs21
B2nc28
B. hayleyi
B. unamae
B. silvatlantica
Non-carried
B. pseudomallei
Chemotaxis assay
Metabolomics
LC-MS/MS
Flow cytometry
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10¹¹
10¹⁰
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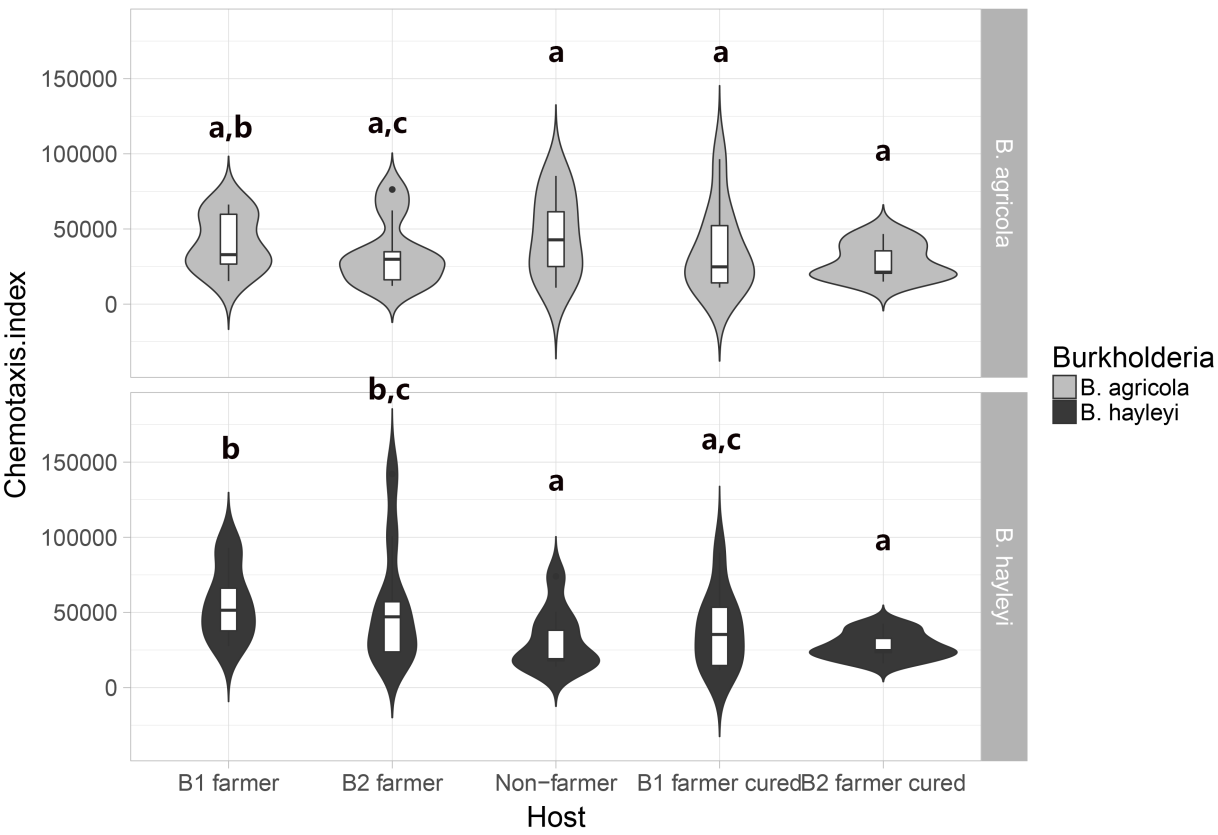
10⁷
Small molecules
P
V
V
P
A



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a,c
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50000
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B. agricola
B. hayleyi
Burkholderia
B. agricola
B. hayleyi
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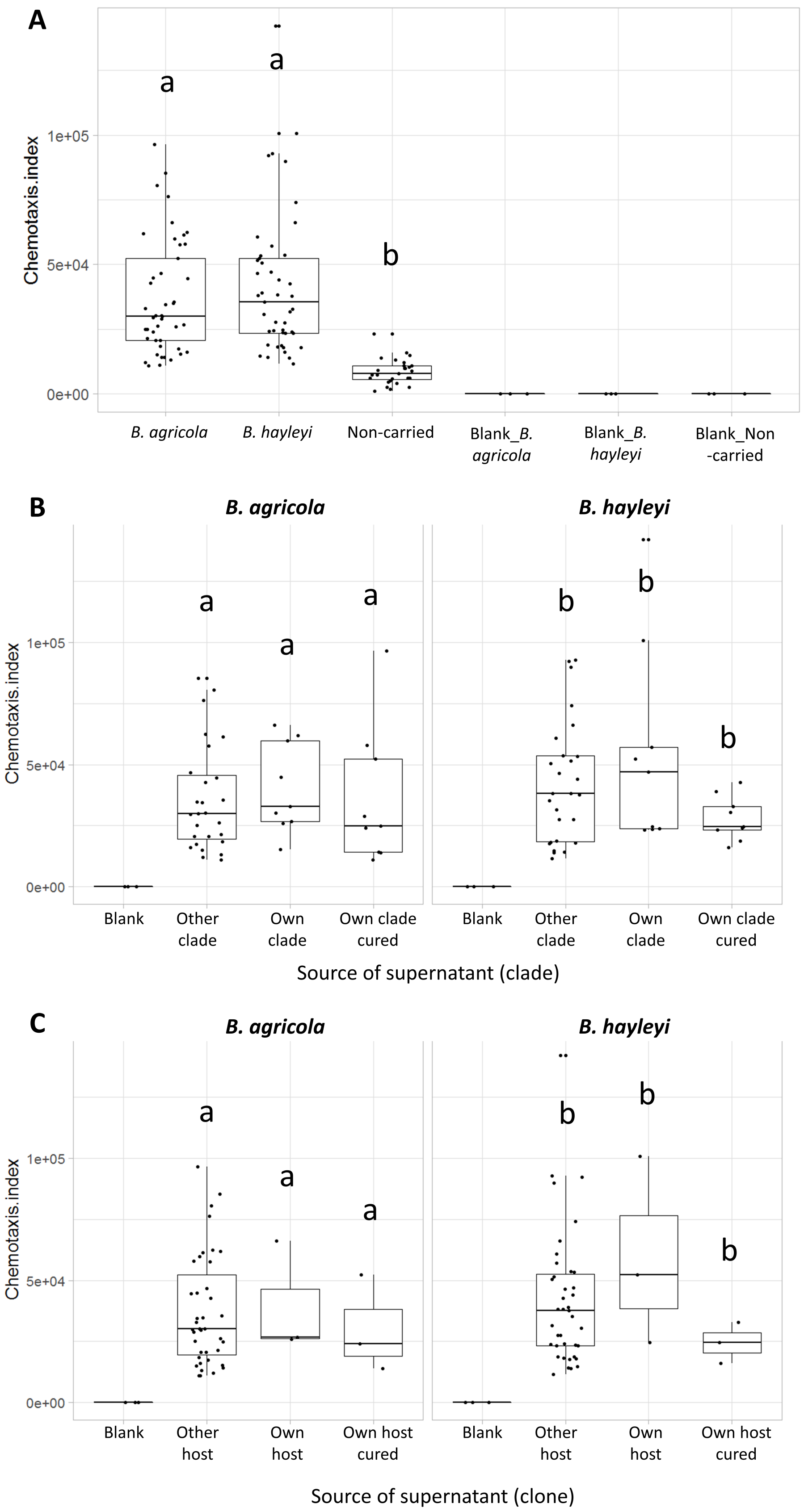
B2 farmer
Non−farmer
B1 farmer cured
B2 farmer cured
Host

832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B. hayleyi
Non-carried
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Blank_B. hayleyi
Blank_Non-carried
B
B. agricola
B. hayleyi
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5e+04
0e+00
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a
a
b
b
b
Blank
Other clade
Own clade
Own clade cured
Blank
Other clade
Own clade
Own clade cured
Source of supernatant (clade)
C
B. agricola
B. hayleyi
Chemotaxis.index
1e+05
5e+04
0e+00
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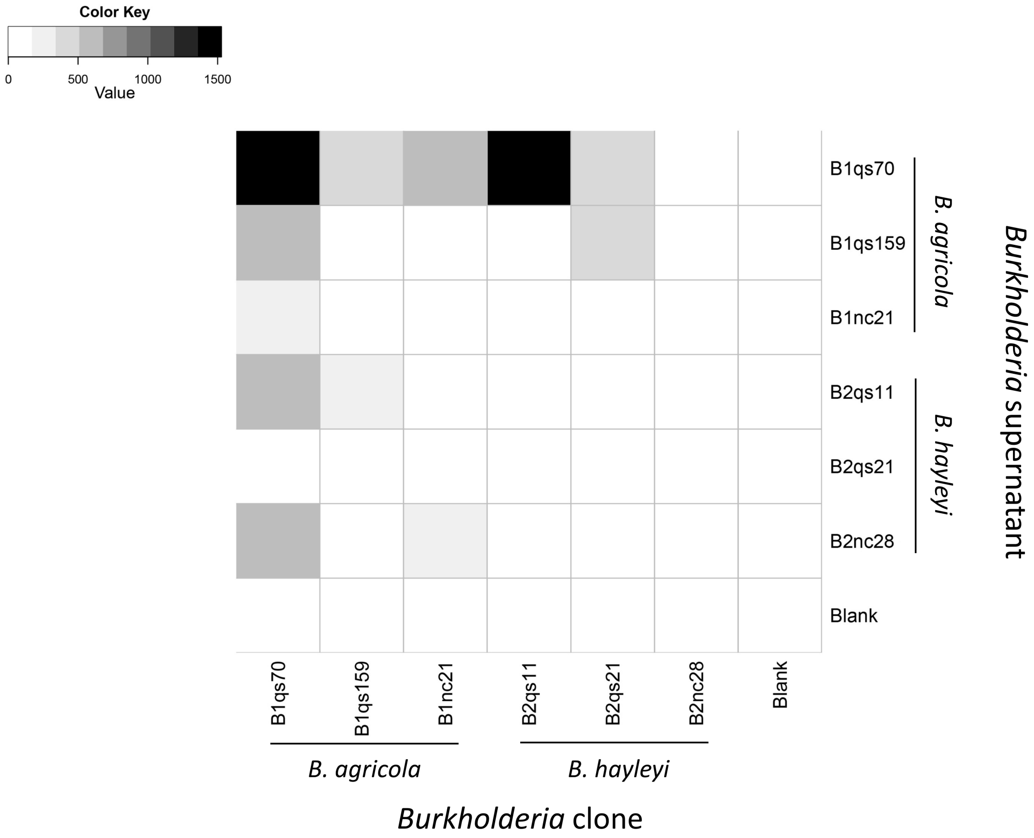
Other host
Own host
Own host cured
Source of supernatant (clone)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B. agricola
B. hayleyi
Burkholderia supernatant
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B2qs21
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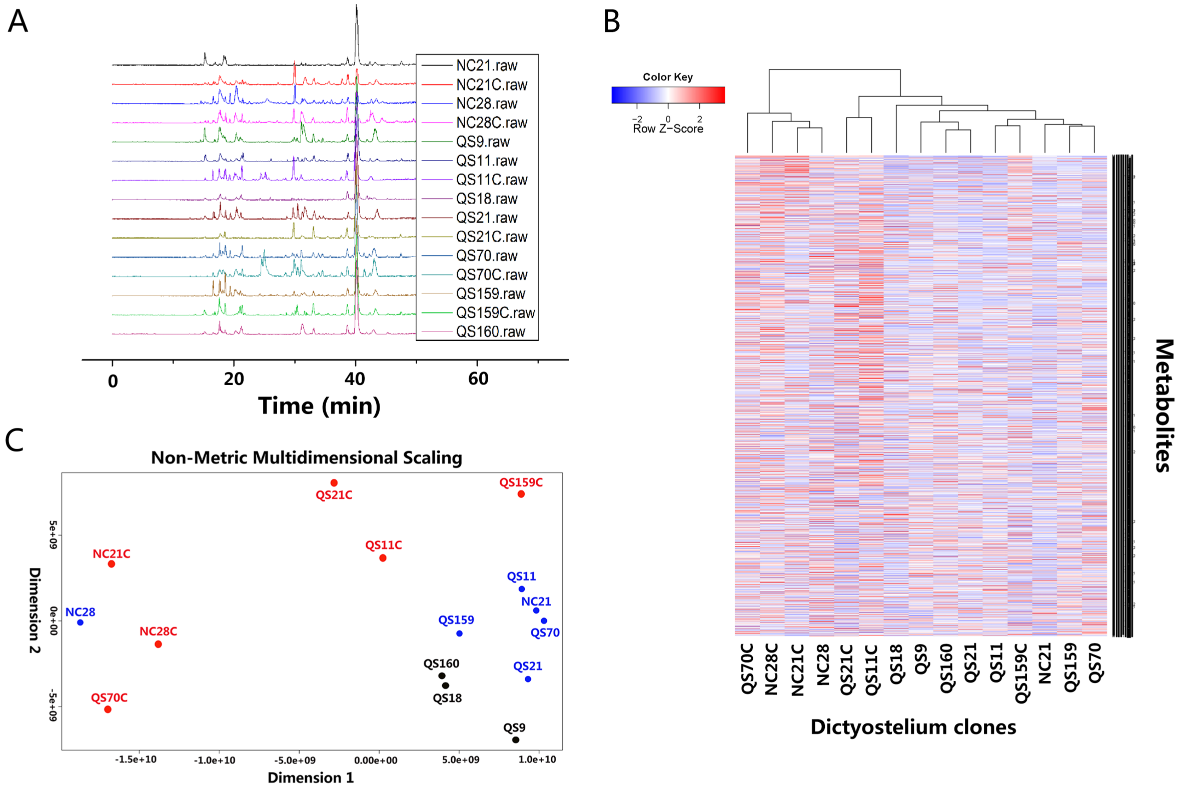
B. agricola
B. hayleyi
Burkholderia clone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QS70C.raw
QS159.raw
QS159C.raw
QS160.raw
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40
60
Time (min)
B
Color Key
-2
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2
Row Z-Score
Metabolites
QS70C
NC28C
NC21C
NC28
QS21C
QS11C
QS18
QS9
QS160
QS21
QS11
QS159C
NC21
QS159
QS70
Dictyostelium clones
C
Non-Metric Multidimensional Scaling
QS21C
QS159C
QS11C
NC21C
QS11
NC21
NC28
QS159
QS70
NC28C
QS160
QS21
QS18
QS70C
QS9
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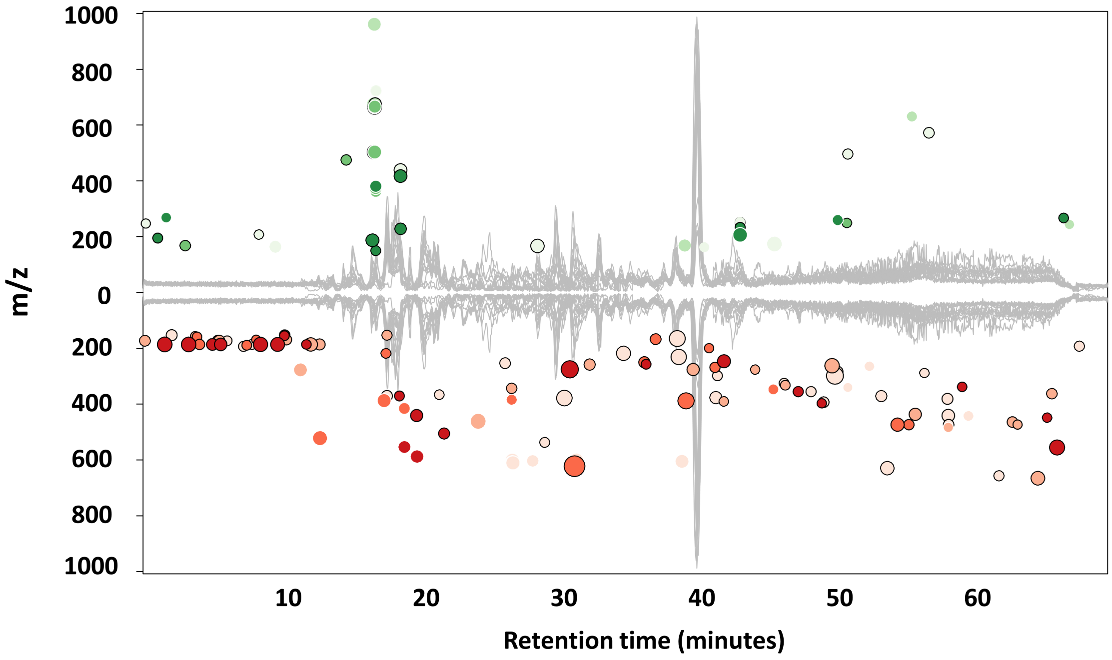
1000
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m/z
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40
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60
Retention time (minutes)