Experimental demonstration that screening can enable the environmental recruitment of a defensive microbiome

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Abstract

Many animals and plants recruit beneficial microbes from the environment, enhancing their defence against pathogens. However, we have only a limited understanding of the assembly mechanisms involved. A game-theoretical concept from economics, *screening,* potentially explains how a host can selectively recruit antibiotic-producing microbes from the environment, by fomenting and biasing competition among potential symbionts in such a way that the likely winners are mutualists. The cuticular microbiomes of *Acromyrmex* leaf-cutting ants inspired one of the first applications of screening theory to mutualisms, and here we use inoculation experiments to test the efficacy of screening *in vitro.* Using agar infused with antibacterial metabolites from the ants' vertically transmitted *Pseudonocardia* symbionts, we show that secondary antibiotic-producing bacteria have higher growth rates than do non-producer strains and are more likely to win in direct competition. Our results demonstrate how game-theoretical concepts from economics can provide powerful insight into host-microbiome coevolution.

Introduction

Many, perhaps most, animal and plant species recruit multiple strains of beneficial microbes from the environment, with one of the most common benefits being defence against pathogens (Kaltenpoth 2009; Barke *et al.* 2011; Seipke *et al.* 2012; Antwis *et al.* 2015; Loudon *et al.* 2016; Duarte *et al.* 2018; Engl *et al.* 2018). It is widely expected that diverse microbiomes provide a more reliable defence against pathogens than do single-species microbiomes, by allowing the equivalent of multi-drug therapy (reviews in Scheuring & Yu 2012; Seipke *et al.* 2012; Antwis & Harrison 2018; Duarte *et al.* 2018; Engl *et al.* 2018). However, despite an abundance of recent

studies, two recent reviews still conclude that "our ability to make predictions about these dynamic, highly complex communities is limited" (Hoye & Fenton 2018), and that "integration between theory and experiments is a crucial 'missing link' in current microbial ecology" (Widder *et al.* 2016).

The Problem of Hidden Information. – We have previously shown that a game-theoretical approach provides a powerful shortcut to modelling the coevolution of host-microbiome assemblies. Specifically, screening theory from economics (Archetti *et al.* 2011a; b; Scheuring & Yu 2012) conceptualises symbioses as a Principal-Agent game (Edwards *et al.* 2006; Weyl *et al.* 2010), in which a Principal (host) tries to recruit one or more suitable Agents (symbionts) out of all possible Agents (e.g. all microbes in the environment), some or most of which are unsuitable. The challenge is that the Principal does not know which Agents are suitable – that is, *the Agent's characteristics are hidden*. This is the Problem of Hidden Information. The equivalent statement in evolutionary biology is that in horizontally acquired symbioses, a host is under selection to recruit mutualistic symbionts out of a species pool that includes both commensals and parasites, but the host is faced with the Partner Choice problem of not being able to recognise which species are mutualistic (Bull & Rice 1991).

Principal-Agent theory provides two solutions to the Problem of Hidden Information: *signalling* and *screening* (Archetti *et al.* 2011a; b). Honest signalling uses the display of costly phenotypes to reveal the quality of potential partners (Spence 1973; Grafen 1990), but it is difficult to envision a cost that can signal cooperativeness *per se* (but see Archetti et al. 2016). Also, even if the host is able to discern symbiont qualities, the host might be unable to actively choose amongst the symbionts. Both problems apply to the recruitment of defensive symbionts where the host cannot use *signalling* because the host has no mechanism for actively choosing amongst symbiont lineages,

cannot identify their characteristics before establishment, or both (Scheuring & Yu 2012).

Here, we present a set of *in vitro* experiments to demonstrate that ant hosts can instead use *screening* to selectively take up antibiotic-producing microbes from the pool of bacteria in the environment. First, we recap how screening works.

Screening. – Imagine a host faced with multiple potential symbionts differing in their benefit to the host. For simplicity, we assume two types, mutualistic and parasitic, where the latter includes commensals because they can impose opportunity costs on hosts (Yu 2001). It is possible for the host to selectively 'screen-in' mutualistic symbionts, provided that the host evolves a 'demanding environment', which imposes a cost on colonising symbionts that is easier to endure if they are mutualists. The benefit of enduring the cost is a host-provided reward that is high enough for the mutualist to enjoy a net benefit (see Box 1 for a human example).

Screening succeeds if the nature and size of the cost and benefit evolve so that only the mutualist reaps a sufficiently large net benefit for natural selection to reward colonisation of the host over any alternatives, including free living. Multiple screening mechanisms appear to exist in nature (Archetti *et al.* 2011a; b; see also Ranger *et al.* 2018). In particular, we have proposed that hosts evolve to foment competition among potential symbionts in such a way that the winners have a high probability of being mutualists, which we call *competition-based screening*. A good example is given by Heil (2013), who studied *Acacia* ant-plants, which can be colonised by ant species that either protect their hostplants (mutualists) or not (parasites). *Acacia* species that provide high amounts of food and housing to incipient ant colonies promote colony growth and worker activity, and the colony that wins the hostplant is the one whose workers kill off the other incipient colonies. Having

numerous, aggressive workers is also the defining characteristic of a mutualistic colony, since the workers attack herbivores. Thus, the demanding environment is interference competition amongst ants, fueled by the hostplant, and the ant species that are best able to endure this cost are the aggressive ones, which is correlated with their mutualistic service to the plant. The benefit of enduring the demanding environment is the high levels of plant-provided reward. This benefit, minus the cost of the risk of colony death due to fighting, is presumably greater than the alternative fitness from living in other microhabitats. What makes this an especially satisfying example is that there also exist *Acacia* species that provide low amounts of food and housing, and these species are regularly colonised by parasitic, non-defending ant colonies, whereas high-reward *Acacia* species are rarely colonised by parasitic ants.

Box 1. A human example of a successful screening mechanism

Any automobile-breakdown insurance company is faced with a *hidden information* problem. Potential clients differ in the probability that their cars will need rescue. Owners of poor-quality cars are more willing to purchase insurance but will impose higher costs on insurers with their more frequent callouts. Owners of high-quality cars are less willing to pay for insurance but would be more profitable to insurers. The challenge for insurers is to find a way to charge a higher price to owners of poor-quality cars and a lower price to owners of high-quality cars, without needing to inspect the cars.

One solution can be viewed at www.rac.co.uk/breakdown-cover (accessed 30 May 2018). Adding the 'At home cover' rescue option costs an additional £5/month, nearly doubling the cost of the cheapest cover at £5.50/month. Poor-quality cars run a nontrivial risk of failing to start in the morning. For high-quality cars, this risk is

negligible. In screening terms, the absence of 'At Home' recovery is *a burden that owners of high-quality cars are better able to endure*. If priced and designed correctly, the two types of owners will voluntarily sign up for the two different coverage levels.

A similar design challenge applies to costly, honest signalling, in which the signal has to be costly in a way that reveals a specific hidden quality. For instance, an expensive car is good at signalling wealth and poor at signalling fidelity or niceness.

Attine defensive microbiomes. – We have argued that competition-based screening is consistent with several biological details of the defensive cuticular microbiomes of attine fungus-growing ants (Scheuring & Yu 2012). These microbiomes produce antibiotics that can control specialised crop diseases, in particular mycopathogens of the genus *Escovopsis* (Currie *et al.* 2006), and may also provide brood hygienic protection (Mattoso *et al.* 2012). They are dominated by Actinobacteria, which are renowned as antibiotic producers (Kaltenpoth 2009; Barka *et al.* 2016; Worsley *et al.* 2018), but only species of the actinobacterial genus *Pseudonocardia* are documented to be vertically transmitted and sufficiently well-studied to qualify as a co-evolving symbiont of attine ants (Cafaro *et al.* 2011; Andersen *et al.* 2013). Other detected actinobacterial strains (a less restrictive term used in preference to 'species' in microbiology) might also be resident but are horizontally acquired, or alternatively might be transient and provide no benefits. Although the relevance of these two transmission routes is not in doubt, their relative importance has been extensively debated without consensus (Sen *et al.* 2009; Cafaro *et al.* 2011; Mueller 2012).

Scheuring and Yu's (2012) model tried to resolve this debate by pointing out that the *Pseudonocardia* biofilm coats the worker surface in antibacterial compounds,

rendering this a demanding environment that we expect antibiotic-resistant strains to be best able to endure. This is because antibiotic-producing bacteria must also have antibiotic resistance, or they would commit suicide when producing antibacterial compounds. Thus, we expect the quality that allows potential symbionts to endure this demanding environment (resistance to antibacterials) to be strongly correlated with the quality that makes them mutualistic (antibiotic production). Attine biology supports the assumption that the demanding environment is paired with high rewards, because the genus-level lineages that host actinobacterial biofilms appear to feed them with subcuticular exocrine glands (Currie *et al.* 2006) – although the secretions of these glands have not yet been characterised.

Screening can potentially explain two other aspects of attine ant biology. Firstly, in the crown-group genus *Acromyrmex*, newly hatched workers are inoculated with *Pseudonocardia* by their sister workers (Poulsen *et al.* 2003b; Andersen *et al.* 2013; Marsh *et al.* 2014), and the monoculture biofilm subsequently blooms over almost the entire worker surface, then retreats to the ant's ventral chest plates after ca. 25 days, when the workers start to forage outside the nest (Poulsen *et al.* 2003a; Andersen *et al.* 2013). At this time it becomes possible to detect the presence of other bacteria on the ant's surface, many of which are also Actinobacteria (Poulsen *et al.* 2005; Andersen *et al.* 2013).

Secondly, Andersen *et al.* (2013) have documented that *Acromyrmex* colonies hosting one of the native *Pseudonocardia* species (*P. octospinosus*, also known as 'Ps1') are colonised by a more diverse secondary cuticular microbiome than are *Acromyrmex* colonies hosting the other species (*P. echinatior*, 'Ps2') (species described in Holmes *et al.* 2016). For simplicity, we refer to these as Ps1 and Ps2. Screening theory predicts that Ps2 imposes the more demanding environment for secondary invaders,

by making stronger or more antibacterials. This would reduce the likelihood of successful colonisation by competitors, but may make the mutualist more costly for the host that provides the resources for its growth. Genomic analysis has confirmed that Ps1 and Ps2 have different sets of secondary metabolite clusters and thus likely produce different spectra of antimicrobial compounds (Holmes *et al.* 2016).

Tests of screening. – Experimental tests of mechanisms that could promote selective recruitment are missing from the microbiome literature (Widder *et al.* 2016; Hoye & Fenton 2018). We address this gap with an *in vitro* model emulating the dynamics on the cuticle of *Acromyrmex* ants. Using *Pseudonocardia* cultures, we created two types of environment on agar media: *demanding* (where growth media are infused with secondary metabolites from *Pseudonocardia*), and *undemanding* (where symbiont metabolites were absent). We then introduced a selection of different bacterial strains representing two classes of potential colonisers from the environment – a set of antibiotic-producing Actinobacteria (*producers*) and a set of bacteria that do not produce antibiotics (*non-producers*) – and we tested the prediction that *producers* are better invaders (by measuring growth rates in the demanding environment) than are *non-producers*. We also staged direct-competition experiments between *producers* and *non-producers* on both *demanding* and *undemanding* media.

We show that antibiotic-*producers* grow relatively more quickly on *demanding* (toxic) media despite growing relatively more slowly on *undemanding* (non-toxic) media, as predicted by screening theory. This result also parsimoniously explains the outcome of the direct-competition experiment, in which antibiotic-*producers* are superior competitors on toxic media, while *non-producers* are superior competitors on non-toxic media. Finally, we also confirm the assumption that antibiotic-*producers*

are more resistant to antibiotics and the prediction that *Pseudonocardia* Ps2 strains are stronger growth-suppressors of other bacteria.

Materials & Methods

We obtained:

(**1**) a set of *Pseudonocardia* Ps1 and Ps2 strains to grow on agar plates, after which we flipped the agar to expose a surface that was open for colonisation but infused with *Pseudonocardia* metabolites. We and others have shown that *Pseudonocardia* releases antibacterials and antifungals when grown in culture (Barke *et al.* 2010; Holmes *et al.* 2016; Van Arnam *et al.* 2016);

(**2**) a set of 'environmental' *antibiotic-producing* bacterial strains, defined as not having an evolutionary history of growing on the cuticle of *Acromyrmex* ants;

(**3**) a set of 'environmental' *non-antibiotic-producing* bacterial strains, defined as not having an evolutionary history of growing on the cuticle of *Acromyrmex* ants; and

(**4**) a set of bacterial strains that reside in the cuticular microbiomes of *Acromyrmex* workers while being *non-antibiotic-producing*, which in theory should preclude their survival in cuticular biofims. We asked why these bacteria do not competitively exclude the resident antibiotic-*producers*, since they are presumably resistant to *Pseudonocardia* metabolites but do not pay the cost of producing antibiotics. We use the term 'resident' only to indicate that we isolated these strains from *Acromyrmex* cuticles; we do not imply that these strains are true native residents like *Pseudonocardia*.

For bacterial growth media, we used Soya Flour + Mannitol (SFM) agar (20g soya flour, 20g mannitol, 20g agar in 1L tap water) and Lysogeny Broth (LB) Lennox (10g tryptone, 5g yeast extract, 5g NaCl in 1L deionised water).

Collection and isolation of bacterial strains

(**1**) Nineteen Ps1 and Ps2 strains were isolated from the cuticles of individual workers of the leaf-cutting ant *Acromyrmex echinatior* (Hymenoptera, Formicidae, Attini). The 19 colonies of these workers were collected in Soberania National Park, Panama, between 2001 and 2014, and reared in climate-controlled rooms at the University of Copenhagen at ca. 25 ºC and 70% relative humidity. The propleural chest plates of individual ants – where the white *Pseudonocardia* biofilms grows most conspicuously (Poulsen *et al.* 2003b; Currie *et al.* 2006) – were scraped with a sterile needle under a stereomicroscope and immediately streak-inoculated across Lennox (LB) media under sterile conditions. The plates were then incubated at 30 °C until visible growth of distinctive white 'cotton ball' colonies of *Pseudonocardia* were clearly apparent. These individual colonies were removed under sterile (LAF bench) conditions and inoculated onto fresh plates, a process that was repeated until clean *Pseudonocardia* cultures were isolated. The *Pseudonocardia* isolates were then grown on Mannitol Soya Flour (SFM) media (optimal for actinobacterial growth and spore production), from which spore stock solutions were prepared in 20% glycerol and kept at -20 °C until use. Each isolate was genotyped to Ps1 or Ps2 (Supplementary Information S1). Five strains from each of these two species have previously been genome-sequenced, formally described, and functionally characterised as different *Pseudonocardia* species by Holmes et al. (2016): *P. octospinosus* (Ps1) and *P. echinatior* (Ps2).

(**2**) The 10 environmental antibiotic-producing strains (all *Streptomyces*) were taken from general collections in the Hutchings lab (Tables 1, S2).

(**3**) The 10 environmental non-antibiotic-producing strains were obtained from the Hutchings lab (2 strains) and from the ESKAPE suite (8 strains) – a set with varying origins (human skin, soil, etc.) associated with hospital-acquired infections and used to test antibiotic resistance or efficacy in clinical/research settings (Tables 1, S2).

(**4**) The 10 *Acromyrmex*-resident, non-antibiotic-producing strains were isolated from *A. echinatior* and *A. octospinosus* ants by culturing from cuticular washes or from the *Pseudonocardia* isolations above. These strains were 16S-genotyped and where possible assigned to genus level by comparison to the NCBI 16S RefSeq database (Supplementary Information S1, Tables 1, S2).

Table 1. The bacterial strains used in our experiments: the 19 *Pseudonocardia* strains isolated from *Acromyrmex echinatior* (columns 1 and 2); the 10 environmental *antibiotic-producer* strains (column 3); the 10 environmental *antibiotic-non-producer* strains (column 4); and the 10 *Acromyrmex-resident, antibiotic- non-producer* strains (column 5). Details for all strains not isolated from *Acromyrmex* in Table S2.

8 *Pseudonocardia* Ps1 strains (colony of origin)	11 *Pseudonocardia* Ps2 strains (colony of origin)	10 environmental antibiotic-producing strains (all *Streptomyces*)	10 environmental non-producer strains	10 *Acromyrmex*-resident non-producer strains
Ae356*	Ae406*	S1. *Streptomyces* M1146	St1. *Escherichia coli*	Sr1. *Ochrobactrum sp.*
Ae263*	Ae160	S2. *S. lividans*	St2. *Lysobacter antibioticus*	Sr2. *Erwinia* sp.
Ae322	Ae505*	S3. *S. coelicolor*	St3. *Bacillus subtilis*	Sr3. *Acinetobacter* sp.
Ae150a*	Ae717*	S4. *S. scabies*	St4. *Pseudomonas putida*	Sr4. *Sphingobacterium sp.*
Ae168*	Ae703	S5. *S. venezuelae*	St5. *Erwinia caratova*	Sr5. *Acinetobacter* sp.
Ae707-CP-A2*	Ae702	S6. *S.* Ae150A-B1	St6. *Enterobacter aerogenes*	Sr6. *Luteibacter sp.*
Ae712†	Ae707	S7. *S.* Ae356-S1	St7. *Acinetobacter baylyi*	Sr7. *Flavobacterium sp.*
Ae280†	Ae331*	S8. *S.* KY5	St8. *Staphylococcus epidermidis*	Sr8. *Brevundimonas sp.*
-	Ae706*	S9. *S.* S4	St9. *Micrococcus luteus*	Sr9. *Acinetobacter sp.*
-	Ae704	S10. *S.* Amy1	St10. *Serratia* KY15	Sr10. *Brachybacterium sp.*
-	Ae715	-	-	-

* *Pseudonocardia* strains that have been genome-sequenced (Holmes *et al.* 2016)
† *Pseudonocardia* strains that were only used in the growth-rate experiment with *Acromyrmex*-resident *non-producers*.

Individual growth-rate experiments

We measured bacterial colony growth rates by growing all *producer* and *non-producer* strains on antibiotic-infused and control media.

To create the antibiotic-infused media, we grew lawns of the 19 *Pseudonocardia* isolates (Table 1), plating 30 µl on 90 mm SFM agar plates. The control plates were inoculated with glycerol only (20%; identical to that used for making solutions from

Pseudonocardia strains). We incubated these plates at 30 °C for 6 weeks, which ultimately produced lawns from 17 strains that could be included in the experiments (6 Ps1 and 11 Ps2 strains). Once each plate was fully (not necessarily uniformly) covered, we flipped the agar to reveal a surface open for colonisation.

The sets of 10 environmental antibiotic-*producers* (all *Streptomyces* spp.) and of 10 environmental *non-producers* (Table 1) were inoculated onto the different media types. Each agar plate was inoculated with 10 evenly spaced colonies, with 3 replicates, generating 2 invader types x 17 Ps-media-types x 3 replicates = 102 Ps1/2-infused plates and 2 x 3 = 6 control plates, i.e. a total of 1020 treatment inoculations and 60 control inoculations. Each strain inoculation used 5 µl of solution (at approximately 1 x 10^6 cells per ml in 20% glycerol), spotted at evenly spaced positions and without coming into direct contact. All plates were incubated at 30 °C for five days, after which photographs were taken under sterile conditions.

Images were processed in Fiji software (Schindelin *et al.* 2012; Rueden *et al.* 2017), creating binary negatives (black & white) so automated tools could identify discrete areas of growth (regions in black) and measure growth areas for each invading strain; in the few cases where binary image resolution was insufficient, outlines were added manually before area calculation. 48 *producer*-inoculated and 57 *non-producer*-inoculated treatment measurements were excluded because plate condition had deteriorated to become unscorable or they were contaminated, leaving a final sample size of 1020-48-57= 915 treatment inoculations and 60 controls.

The second growth-rate experiment compared the 10 *Acromyrmex*-resident, *non-producer* strains with 9 of the environmental *producer* strains. All 19 *Pseudonocardia* strains grew sufficiently to be included in this experiment, and each plate was again inoculated with 10 or 9 evenly spaced colonies. Starting sample sizes were therefore 2

312 invader types x 19 Ps-media-types x 3 replicates = 114 Ps1/2-infused plates and 2 x 3
313 = 6 control plates, giving a total of 1083 treatment inoculations and 57 controls. 50
314 *producer* and 20 *non-producer* treatment measurements were excluded for the same
315 reasons as above, giving a final sample of 1083-50-20=1013 treatment inoculations
316 and 57 controls for scoring as described above.

317 *Pairwise competition experiment*

318 Building on the above experiment, where *producer* strains had relatively higher
319 growth rates on *Pseudonocardia*-infused media, we tested whether this growth
320 advantage (plus any direct interference interactions) could result in competitive
321 exclusion of *non-producers*. We co-inoculated pairs of environmental *producers* and
322 *non-producers* on both *Pseudonocardia*-infused media and control media (prepared as
323 in the individual growth-rate experiment), and we measured the outcome of
324 competition as a win, loss, or draw. We predicted that the *producer* strains would be
325 more likely to win when competing on *Pseudonocardia*-infused media.

326 To keep the number of tests manageable, we used only two combinations of
327 *Pseudonocardia* and *Streptomyces*: *Pseudonocardia* Ps1 (strain Ae707) +
328 *Streptomyces* S8 and *Pseudonocardia* Ps2 (strain Ae717) + *Streptomyces* S2 (Table
329 1). These combinations are representative of the results from the growth-rate
330 experiment: the two *Streptomyces* strains grew more slowly than most of the *non-*
331 *producers* on control media and either near the median growth rate of (S8) or faster
332 than (S2) the *non-producers* on *Pseudonocardia*-infused media.

333 We competed *Streptomyces* S8 and S2 against the 10 environmental *non-producer*
334 strains (20 pairings), using spore titrations of S8 and S2 consisting of 10^6 spores per
335 ml for each strain in 20% glycerol. Non-producers were grown overnight in 10 ml

LB-Lennox, subcultured with a 1:100 dilution into a fresh 10 ml LB-Lennox, grown at 37 °C for 3-4 hours, after which OD600 was measured, assuming that OD600 = 1 represented 8×10^9 cells. Similar dilutions of 10^6 cells per ml were made for each *non-producer* strain in 20% glycerol, after which *producer* and *non-producer* dilutions were mixed 1:1. Each pair of *producer* and *non-producer* was co-inoculated as a mixture of 20 µl (10^4 spore-cells of each) on the designated *Pseudonocardia*-infused media (set up as above) with 5 replicates per pairing. We used 150 plates for the S8 experiment (including 100 control plates; 10 replicates per pairing) and 100 plates for the S2 experiment (including 50 control plates; 5 replicates per pairing). Plates were incubated at 30 °C for 5 days and then photographed, after which images were scored with respect to the *producer* as: win (dominant growth), draw (both strains growing with no clear dominant), or loss (little or no visible growth), with reference to images of each strain grown alone on control media to minimise observer bias. One plate's outcome was too ambiguous to score and was omitted. All plates were independently scored by two observers, one using only photos, which produced data sets giving the same statistical results. We report the results from the observer who scored from direct observation.

Antibiotic-resistance profiles

The key assumption of screening theory is that antibiotic-*producers* are better at resisting antibiotics, as measured by growth rates in the presence of antibiotics, because this correlation is what allows *producer* strains to better endure the demanding environment produced by *Pseudonocardia*. We tested this assumption by growing the 10 environmental *producer* strains and the 10 environmental *non-producer* strains (Table 1, S2) in the presence of 8 different antibiotics, representing a range of chemical classes and modes of action. Antibiotics were added to 1 ml of LB-

Lennox medium in a 24-well microtitre plate at 6 different concentrations. The relative concentration range was the same for each antibiotic, although actual concentrations reflected activity (Table S3). *Producers* and *non-producers* were inoculated onto plates and incubated at 30 °C for 7 days, then photographed (Table S3). Lowest Effective Concentration (LEC, lowest concentration with inhibitory effect) and Minimum Inhibitory Concentration (MIC, lowest concentration with no growth) scores were assigned on a Likert scale of 1–6, where 1 was no resistance and 6 was resistance above the concentrations tested (adapted from generalised MIC methods; reviewed by Balouiri *et al.* 2016).

Statistical analyses

Initial models revealed correlated residuals by *Pseudonocardia* strain media and by inoculant strain, so we used mixed-effects models to incorporate strain identities as random intercepts, and we nested plate replicate within the *Pseudonocardia* strains. Statistical significance was determined by term deletion, and final-model residuals were approximately homoscedastic and uncorrelated within random factor levels. Analyses were carried out in *R* 3.4.4 (R Core Team 2017) with *lme4* 1.1-17 (Bates *et al.* 2015), *tidyverse* 1.2.1 (Wickham 2017), and *RColorBrewer* 1.1-2 (www.colorbrewer.org, accessed 4 Mar 2018). R scripts and data are available in Supplementary Information (S4) and at github.com/dougwyu/screening_Innocent_et_al.

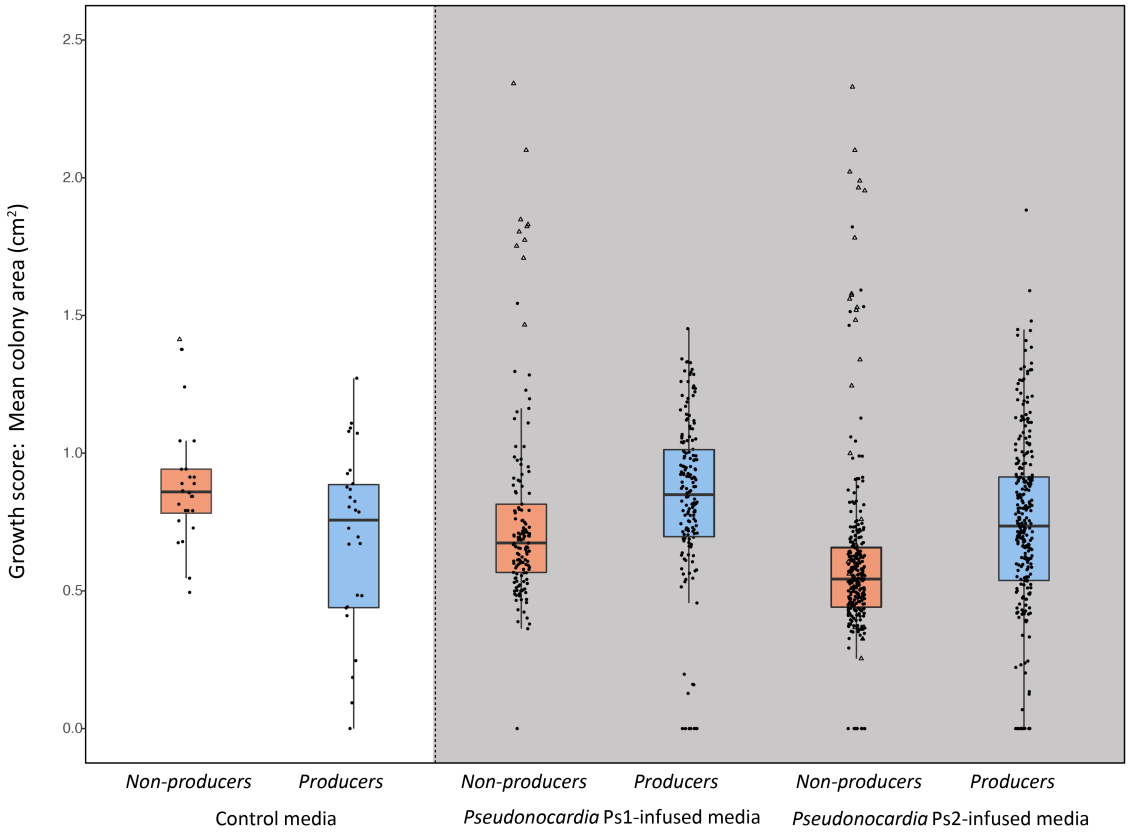
Results

Individual growth-rate experiments. – As predicted by the screening model, *non-producers* grew more quickly on undemanding control media and *producers* had higher growth rates on the demanding *Pseudonocardia*-infused media, producing a

highly significant statistical interaction effect (Fig. 1A). There was also a significant main effect of *Pseudonocardia* genotype, with both *non-producers* and *producers* exhibiting a lower growth rate on Ps2-infused media, relative to Ps1-infused media, consistent with Andersen et al.'s (2013) observation that *Acromyrmex* colonies hosting Ps2-dominated cuticular microbiomes were less prone to secondary invasion by other bacteria.

The resident *non-producers* isolated from cuticular microbiomes had significantly slower growth rates overall, even on control media without antibiotics (Fig. 1B). This suggests that they are unable to outcompete *producer* strains on the cuticle of *Acromyrmex* ants and raises the question why these *non-producer* species can persist at all.

A. Growth rates of environmental *non-producers* vs. *producers*



399

B. Growth rates of *Acromyrmex* resident *non-producers* vs. *producers*

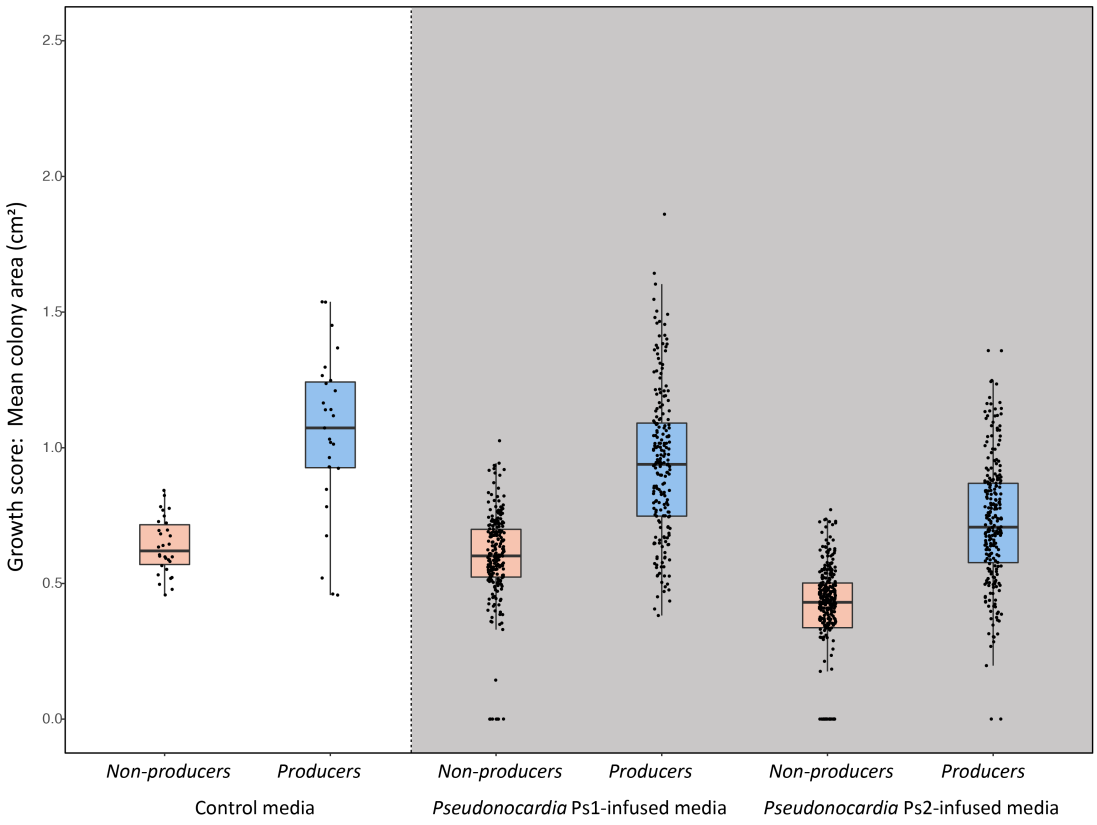


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Figure 1. Individual growth-rate experiments. Bacterial colony sizes after 5 days at 30 ºC, with the boxplots indicating medians ± one quartile. The white section shows growth rates on control media, and the grey section shows growth rates on the *Pseudonocardia*-infused media. Coral boxes represent *non-producer* strains, and blue boxes represent *producer* strains. For analysis, a linear mixed-effects model, including *Pseudonocardia* strain (`Ps.strain`, 17 groups) and inoculated bacterial species (`Inv.strain`, 20 groups) as random intercepts, was used to test for interaction and main effects of growth media (`Control` vs. `Ps1.infused` vs. `Ps2.infused`) and antibiotic production (`Non.producers` vs. `Streptomyces`): (*`lme4::lmer`*`(Growth.score ~ Media * Antibiotic.production + (1|Ps.strain/Plate) + (1|Inv.strain))`). **A.** Environmental strains. There was a highly significant interaction effect, such that environmental non-producer strains grow more rapidly on control media, while *Streptomyces* grow more rapidly on *Pseudonocardia*-infused media (n = 975, χ^2 = 45.86, df = 2, p < 0.0001). There was also a highly significant main effect of *Pseudonocardia* genotype, with growth being lower on Ps2-infused media (n = 915, χ^2 = 24.55, df = 1, p < 0.0001, control-media data omitted for this analysis). One non-producer strain (*Staphylococcus epidermidis*) grew more rapidly than all other strains (open triangle points), demonstrating the need to control for correlated residuals by strain. The y-axis has been truncated at 2.5 for clarity (the full figure is in Supplementary Information). **B.** *Acromyrmex*-resident, *non-producer* strains. There was no interaction effect (χ^2 = 2.64, df = 2, p = 0.27), but both main effects were highly significant. The *non-producers* grow more slowly on all media types (χ^2 = 20.96, df = 1, p < 0.0001), and bacterial growth was generally slower on Ps2-infused media (χ^2 = 21.43, df = 1, p < 0.0001, control-media data omitted).

425 *Pairwise competition experiment*. – The two *producer* strains were much more likely
426 to win in direct competition against the *non-producer* strains when grown on the toxic
427 (*Pseudonocardia*-infused) media (Fig. 2), which demonstrates that growth rates in
428 isolation (Fig. 1A) are sufficient predictors of success in competitive interactions,
429 although other interactions, such as antibiotic production by the *Streptomyces*, likely
430 also contribute. These results support the screening model prediction that a
431 demanding environment (facilitated by a host) can determine the profile of successful
432 colonisers of the cuticular microbiome.

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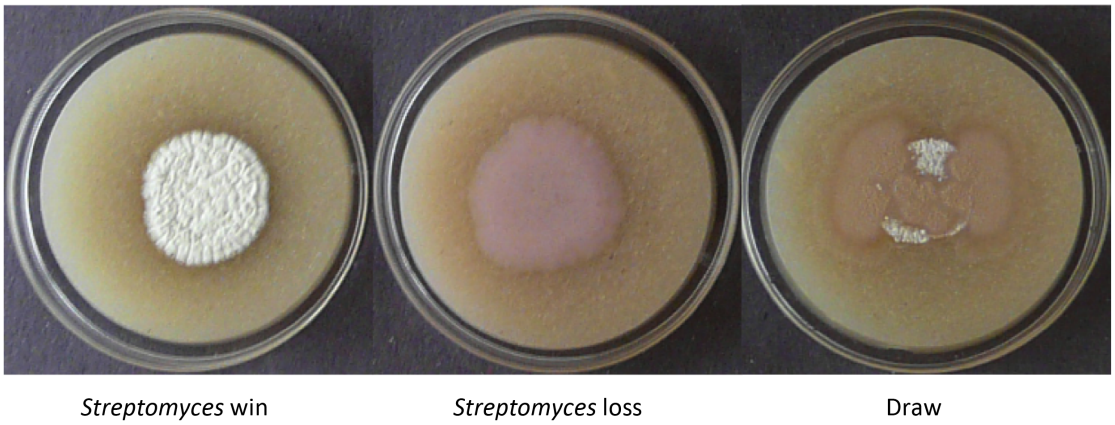

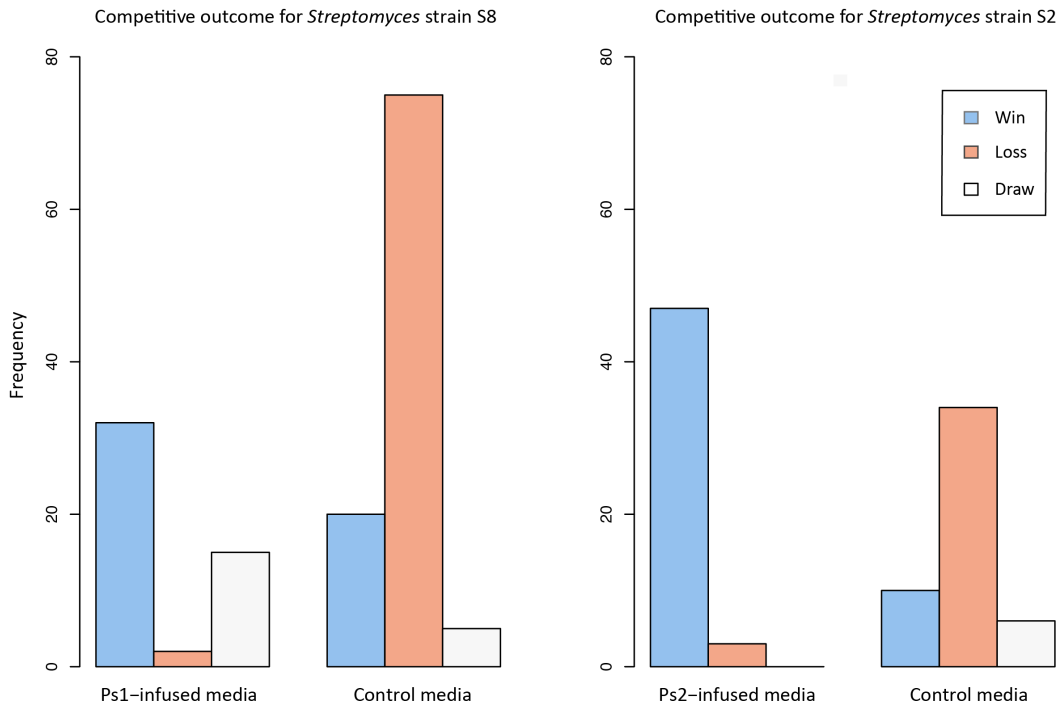

Figure 2. Pairwise competition experiment, scoring whether or not *producers* prevail. **A**. Representative images of agar plates at five days post-inoculation showing examples of the three competitive outcomes: Win (S2 vs. St3 on Ps2 media), Draw (S2 vs. St3 on control media), and Loss (S8 vs. St3 on control media; strain details in Table 1). **B**. Barcharts of competitive outcomes for the two *Streptomyces producer* strains (S8 and S2; Table 1), each pairwise co-inoculated with ten non-antibiotic-producer strains on *Pseudonocardia*-infused media and on control media. For analysis, draw outcomes were omitted, and a general linear mixed-effects model, including non-antibiotic-producer strain as a random intercept

(10 groups), was used to test for an effect of the `medium` term (`Control` vs. `Ps1/2-infused`) on competitive outcome (`Win` vs. `Loss`) ((*`lme4::glmer`*`(outcome ~ medium + (1|non.producer.strain), family = binomial)`). Significance was estimated using term deletion. In both experiments, *producer* strains (*Streptomyces*) were more likely to be competitively superior when grown on the *Pseudonocardia*-infused media (Left: n = 129, χ^2 = 103.6, df = 1, p < 0.0001; Right: n = 94, χ^2 = 87.9, df = 1, p < 0.0001).

The screening model assumes that *producer* strains have greater resistance to antibiotics than do *non-producer* strains. We confirmed this assumption for this experiment because both the Lowest Effective Concentration (LEC, lowest concentration with inhibitory effect) and the Minimum Inhibitory Concentration (MIC, lowest concentration with no growth) were greater for the *producer* strains (Fig. 3). We also observed that the *resident non-producers* had high levels of antibiotic resistance (Fig. 3), as expected (Fig. 1B).

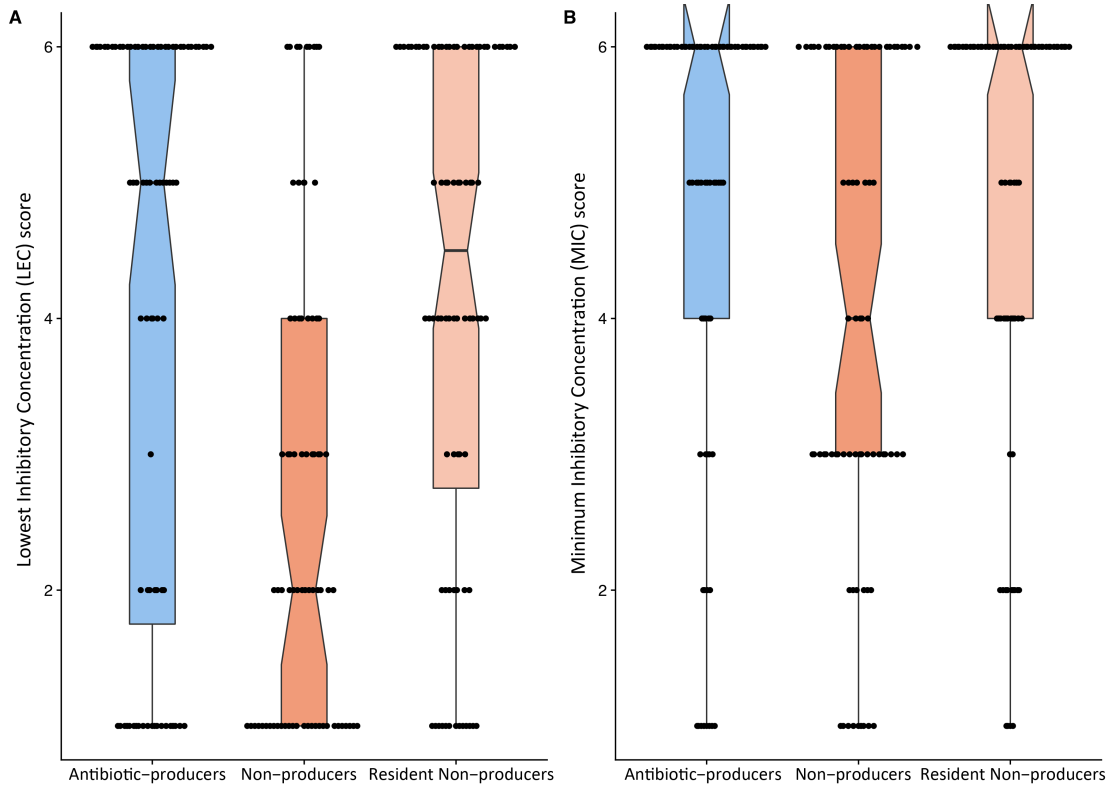

Figure 3. Antibiotic resistance profiles for *producer*, *non-producer*, and *resident non-producer* strains (Table 1). Boxplots indicate medians (notches) ± one quartile. For analysis, we calculated each strain's mean growth score across the eight tested antibiotics (reducing from n = 155 to n = 20; details in S3). *Producers* showed higher levels of resistance than did *non-producers* for both measurements: Wilcoxon two-sided test (`wilcox.test`), W = 94.5, p = 0.0017 for LEC (**A**) and W = 80, p = 0.0253 for MIC (**B**), after correction for two tests (`p.adjust(method="fdr")`). *Producers* and *Resident Non-producers* showed no difference in resistance levels (p = 0.44 and 0.25).

Discussion

We used *in vitro* experiments to test key predictions and assumptions of a screening model (Scheuring & Yu 2012) that was designed to explain how leaf-cutting ants could use a vertically transmitted symbiont, *Pseudonocardia*, to selectively recruit

additional antibiotic-producing bacterial strains from the large pool of potential bacterial symbionts present in the environment.

We showed that *non-producer* invasive strains grow more slowly than do antibiotic-*producer* strains on *Pseudonocardia*-infused media (Fig. 1A). Since the control plates showed that *non-producers* have higher intrinsic growth rates, we attribute the restricted growth on *Pseudonocardia*-infused plates to inhibition by *Pseudonocardia* metabolites, consistent with the large number of antibacterials documented as encoded in the genomes of both Ps1 and Ps2 (Holmes *et al.* 2016). We also showed that Ps2 is a stronger suppressor of bacterial growth in general, consistent with Andersen et al.'s (2013) observation that Ps2-hosting *Acromyrmex* colonies assemble lower-diversity (and more Ps2-dominated) cuticular biofilms on foraging workers. Finally, we confirmed that our *producer* strains (all genus *Streptomyces*) had higher resistance to antibiotics than did our *non-producer* strains (Fig. 3).

Our pairwise competition experiment confirmed that growing on *Pseudonocardia*-infused media renders *producer* strains competitively superior (i.e. they usually win) or at least resistant to exclusion (they more often draw than lose, especially on the less-toxic Ps1-infused media) (Fig. 2). This *in vitro* experiment mimics the conditions we expect to occur on the cuticle of *Acromyrmex* workers after their full-body *Pseudonocardia* biofilm (obtained shortly after hatching) has retreated to the laterocervical chest plates (and possibly into the subcuticular glands), when the workers begin to forage outside the nest, where they are exposed to many bacterial colonists (Poulsen *et al.* 2005; Andersen *et al.* 2013; Marsh *et al.* 2014; Andersen *et al.* 2015).

Taken together, these results show how a single, vertically transmitted, antibiotic-producing symbiont such as *Pseudonocardia* can be used by the ant host to create a

demanding environment on the ant surface that favours the recruitment of a more diverse set of antibiotic-producers. In short, vertical transmission of one mutualist enables the successful horizontal acquisition of other mutualists. Vertical transmission and horizontal symbiont acquisition are therefore not mutually exclusive explanations, consistent with a mixture of both not being uncommon in other symbioses (Ebert 2013; Bourguignon *et al.* 2018; da Costa & Poulsen 2018; Ivens *et al.* 2018). Under some circumstances, mutualistic stability through screening may be able to do without a vertically transmitted symbiont; Duarte et al. (2018) have argued that a host might be able to create a suitably demanding environment by evolving the right set of chemical exudates.

We note that in a colonial social insect, one needs to be precise about symbiont transmission and symbiont inheritance. Since virgin *Acromyrmex* queens are inoculated upon hatching by nurse workers that maintain monocultures of either Ps1 or Ps2, the secondary acquisition of other *producer* symbionts by foraging workers is highly unlikely to disrupt verticality in the inheritance of native *Pseudonocardia* symbionts down the generations.

Acromyrmex-*resident non-producers*. – We also ran a growth-rate experiment with a set of *resident non-producer* strains that had been isolated from *Acromyrmex* ant cuticles (Fig. 1B) and found that they generally had low intrinsic growth rates, making it unlikely that they can competitively exclude *producers*. It seems surprising that we found these *non-producer* strains at all, but we note that the fact that we could isolate them does not imply that they are abundant. Resident *non-producer* taxa are only found on some (not all) field-sampled *Acromyrmex* workers (Andersen *et al.* 2013; Innocent et al., in prep) and generally below 1% prevalence when measured by amplicon read number. They may only be transient environmental contaminants,

since many were isolated via whole-ant washes, or strains persisting in non-equilibrium dynamics. In simulation models (Boza, G., Worsley, S., Yu, D., Scheuring, I., unpubl. data), we have found that if bacterial competition is limited to nearest neighbours and if antibiotics slowly diffuse across the ant surface, even non-antibiotic-resistant strains can sometimes persist for long periods of time.

It remains an open question why three different *Acromyrmex* species in Panama all host the same two *Pseudonocardia* strains, Ps1 and Ps2 (Andersen *et al.* 2013). The genomes of Ps1 and Ps2 strains each encode a single distinct nystatin-like antifungal, plus variable and partially segregated sets of secondary metabolite biosynthetic gene clusters with predicted antibacterial functions (Holmes *et al.* 2016). These two *Pseudonocardia* species are almost never found in the same colony even though they are encountered at approximately equal rates in field colonies (Poulsen *et al.* 2005). The difference between Ps1 and Ps2 in microbiome invasibility (Fig. 1A) may reflect a tradeoff between stronger endogenous production of antimicrobials early in life against the recruitment of a more diverse set of secondary antibiotic producers later in life.

Comparable studies to ours using other species of fungus-growing ants will be needed to test the generalisability of screening to other attine ant lineages that maintain actinobacterial biofilms, including species that appear to have cuticular microbiota with abundant actinomycete genera other than *Pseudonocardia* (Sen *et al.* 2009; Andersen *et al.* 2013; Meirelles *et al.* 2014). Our results suggest that cuticular biofilms can be adequately simulated on agar plates, providing a tractable experimental model with which to test microbiome assembly mechanisms. Such

studies might also shed light on why *Atta* and some other genus-level branches of the attine fungus-growing ants have secondarily lost their cuticular biofilms, a phenomenon that remains poorly understood. The screening requirement to pair a demanding environment with a high reward imposes nontrivial costs on hosts, and in some cases, this cost might have tipped the cost-benefit ratio in favour of chemical pest control over biological control (Fernandez-Marin *et al.* 2009; Fernandez-Marin *et al.* 2015).

Toward a general game theory of hosted microbiomes

Hosted microbiomes are coevolutionary metacommunities in which most of the species interactions are difficult to observe. There are numerous calls to use ecological theory to understand microbiomes (Widder *et al.* 2016; Hoye & Fenton 2018), but we propose that a game theory approach may be more promising for producing testable predictions. Screening (Archetti *et al.* 2011a) and password signalling (Archetti *et al.* 2016) are candidate solutions to the Problem of Hidden Information. When hosts evolve to use screening, the emergence of demanding environments can be thought of as an exercise in applied ecology, where the host evolves a mechanism to foment and bias competition by modifying resource and toxicity levels to encourage cooperative microbial communities (see Foster *et al.* 2017; Duarte *et al.* 2018 for similar perspectives).

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