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 selectively recruits mutualistic microbes from the environment by fomenting and biasing competition among potential symbionts in such a way that the more likely winners are antibiotic producers. The cuticular microbiomes of *Acromyrmex* leaf-cutting ants inspired one of the first applications of screening theory, and here we simulate this system *in vitro* to test screening. On agar infused with antibacterial metabolites from *Acromyrmex*'s vertically transmitted *Pseudonocardia* bacteria, we show that antibiotic-producing *Streptomyces* bacteria exhibit higher growth rates than do non-antibiotic-producer strains and are more likely to win in direct competition. Our results demonstrate how game-theoretical concepts 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 application 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 studies on

microbiomes, 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 game theory from economics provides a powerful shortcut to modelling the coevolution of hosts and their symbionts (Edwards *et al.* 2006; Weyl *et al.* 2010; Archetti *et al*. 2011a). Specifically, a host can be conceptualised as a 'Principal' that 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 (Archetti *et al.* 2011b; Scheuring & Yu 2012). The challenge is that the Principal does not know which Agents are suitable – that is, *the Agent's characteristics are hidden*. This is known as the Problem of Hidden Information in economics. The equivalent statement in biology is that in horizontally transmitted symbioses, a host is under selection to recruit mutualistic symbionts out of a species pool that also contains commensals and parasites, but the host is faced with the Partner Choice problem (Bull & Rice 1991) of not being able to recognise which species are mutualistic.

Game theory provides two solutions to the Problem of Hidden Information: *signalling* and *screening* (Archetti *et al.* 2011a; b). Signalling uses the display of costly phenotypes to honestly reveal the quality of potential partners (Spence 1973; Grafen 1990), but it is difficult to envision a cost that can signal cooperativeness *per se* (although 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 it cannot differentiate symbiont lineages before establishment, or has no mechanism for choosing amongst lineages, or both.

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

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 S5 for a human example of screening.)

Multiple screening mechanisms appear to exist in nature (Archetti *et al.* 2011a; b; see also Ranger *et al.* 2018), and in particular, we have proposed an elaboration of screening in which hosts evolve to foment and bias competition among potential symbionts in such a way that mutualists enjoy a higher probability of being the winners, which we call *competition-based screening* (Archetti *et al.* 2011b). 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 provide abundant food and housing to incipient ant colonies to 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 mutualist, since

112 such workers attack herbivores. Thus, *the demanding environment is interference*
113 *competition amongst the ants themselves*, fueled by the hostplant, and the ants best
114 able to endure this cost are the aggressive, ergo mutualistic, species. The benefit of
115 enduring the demanding environment is the high levels of plant-provided reward. This
116 fitness gain, minus the cost of the risk of colony death due to fighting, is presumably
117 greater than the fitness gained by living elsewhere. What makes this an especially
118 satisfying example is that there also exist *Acacia* species that provide low amounts of
119 food and housing, which are regularly colonised by timid, non-defending ants,
120 whereas high-reward *Acacia* species are usually colonised by aggressive ants.

121 *Attine defensive microbiomes and the Red Queen*. – We have argued that competition-
122 based screening is consistent with several biological details of the defensive cuticular
123 microbiomes of attine fungus-growing ants (Scheuring & Yu 2012). These
124 microbiomes produce antibiotics that can control specialised crop diseases, in
125 particular mycopathogens in the genus *Escovopsis* (Currie *et al.* 2006), and may also
126 provide brood hygienic protection (Mattoso *et al.* 2012). By biomass, the dominant
127 actinobacterial constituent of attine cuticular microbiomes is the genus
128 *Pseudonocardia*, and there is considerable evidence that attine ants have maintained a
129 long-term, coevolved, and mutualistic relationship with *Pseudonocardia*, via vertical
130 transmission (Cafaro *et al.* 2011; Andersen *et al.* 2013; Steffan *et al*. 2015; Li *et al*.
131 2018). However, it is recognised that an exclusive relationship between an ant lineage
132 and its vertically transmitted bacterial lineage raises the Red Queen problem (Currie
133 et al. 2006): how can *Pseudonocardia* maintain effectiveness against *Escovopsis*
134 *weberi*, which reproduces sexually and has evolved countermeasures in the form of
135 two metabolites that are upregulated after infecting the attine fungal cultivar:
136 *melinacidin* and *shearinine*. Both these virulence factors are potent growth

137 suppressors of the *Pseudonocardia* strains (a less restrictive term used in preference
138 to 'species' in microbiology) that are hosted by *Acromyrmex* ants, and shearinine also
139 paralyses and kills *Acromyrmex echinatior* workers (Heine et al. 2018).

140 *Multi-species defensive microbiomes*. – Multi-species defensive microbiomes are an
141 obvious candidate solution to the Red Queen problem, allowing ants to apply multi-
142 drug therapy against *Escovopsis*. Multiple amplicon and culture studies (Kost et al.
143 2007; Haeder et al. 2009; Sen et al. 2009; Barke et al. 2010; Schoenian et al. 2011,
144 Andersen et al. 2013) have documented the presence of actinomycete genera such as
145 *Streptomyces* and *Amycolatopsis*, which are well-known antibiotic producers
146 (Kaltenpoth 2009; Barka *et al.* 2016; Worsley *et al.* 2018), including an amplicon
147 study reporting *Streptomyces* read frequencies of >5% on eleven field-collected
148 *Acromyrmex echinatior* workers and *Amycolatopsis* read frequencies of 35% and 99%
149 on two *Trachymyrmex zeteki* workers (Andersen et al. 2013). These strains are almost
150 certainly acquired horizontally (i.e. from the environment), but presence alone does
151 not demonstrate that these strains are live and contributing antibiotics. However,
152 Schoenian *et al*. (2011) used liquid-chromatography-mass-spectrometry (LC-MS) to
153 demonstrate that *Streptomyces* cultured from the cuticles of *Acromyrmex* workers
154 (Haeder et al. 2009) synthesise antifungal and antibacterial valinomycins, antimycins,
155 actinomycins, and candicidins, which act synergistically *in vitro* to inhibit *Escovopsis*,
156 thus demonstrating the possibility of multi-drug therapy. Crucially, Schoenian *et al*.
157 also used MALDI imaging to visualise up to several nanograms of valinomycin on the
158 cuticles of *Acromyrmex echinatior* workers, thus demonstrating *in vivo* metabolic
159 activity of *Streptomyces*. Also, Worsley *et al*. (S. Worsley *et al*., *unpubl. data*) have
160 recently used stable isotope probing followed by RNA-sequencing (SIP-RNA) of
161 cuticular bacteria isolated from *Acromyrmex echinatior* workers that had been fed

162 ^{13}C-glucose solution to document the uptake of ^{13}C by twelve bacterial genera,
163 including *Pseudonocardia* and *Streptomyces*, representing ~40% and ~20% of
164 sequence reads, respectively. In sum, the weight of evidence is shifting toward the
165 conclusion that (at least) *Acromyrmex* recruits and supports a multi-species defensive
166 microbiome, as has been reported for other taxa (see *Introduction*). This now raises
167 the question of why *Acromyrmex* continues to host *Pseudonocardia*.

168 *Screening applied to the attine microbiome.* – Newly hatched *Acromyrmex* workers
169 are inoculated with *Pseudonocardia* by their sisters (Poulsen *et al.* 2003b; Andersen
170 *et al.* 2013; Marsh *et al.* 2014), and the monoculture blooms over the workers, then
171 retreats to the propleural plates of the ventral surface after ~25 days, when the
172 workers start foraging (Poulsen *et al.* 2003a; Andersen *et al.* 2013). This is when
173 other actinomycete bacteria can be detected on the ant.

174 Scheuring and Yu's (2012) model argues that because *Pseudonocardia* coats the
175 worker cuticle with antibacterials, the cuticle is rendered a demanding environment
176 that we expect antibiotic-producing strains to be better able to endure. This is because
177 antibiotic-producing bacteria must also have antibiotic resistance, or they would
178 commit suicide when producing antibacterials. Thus, the quality that allows potential
179 symbionts to endure this demanding environment (resistance to antibacterials) is
180 correlated with the quality that makes them mutualistic (antibiotic production). This
181 demanding environment is paired with high rewards from the ant's exocrine glands
182 (Currie *et al.* 2006; Li et al. 2018; S. Worsley *et al*. unpubl data), and the combination
183 is predicted to foment and bias competition in favour of strains that can produce the
184 most effective antibiotics (Scheuring & Yu 2012).

185 Screening is potentially consistent with an interesting complication discovered by
186 Andersen *et al*. (2013), who showed that *Acromyrmex* colonies hosting one of the

187 native *Pseudonocardia* strains (*P. octospinosus*, also known as 'Ps1') are colonised
188 by a more diverse secondary cuticular microbiome than are *Acromyrmex* colonies
189 hosting the other strain (*P. echinatior*, 'Ps2') (strains described in Holmes *et al.*
190 2016). For simplicity, we refer to these as Ps1 and Ps2. Genome analysis has
191 confirmed that Ps1 and Ps2 code for different sets of secondary metabolite clusters
192 (Holmes *et al.* 2016). Screening theory predicts that Ps2 imposes the more demanding
193 environment, by making stronger or more antibacterials, and thus reducing the
194 likelihood of successful secondary colonisation.

195 *Tests of screening*. – Experimental tests of mechanisms that could promote selective
196 recruitment are missing from the microbiome literature (Widder *et al.* 2016; Hoye &
197 Fenton 2018). We address this gap with an experiment that tests screening *in vitro.*
198 Using *Pseudonocardia* cultures, we created two types of environment on agar media,
199 emulating the cuticle of *Acromyrmex* ants: *demanding* (where the agar was infused
200 with metabolites from *Pseudonocardia*), and *undemanding* (where *Pseudonocardia*
201 metabolites were absent). We then introduced a variety of bacterial strains
202 representing two classes of colonisers – antibiotic-producing *Streptomyces*
203 (*producers*) and bacteria that do not produce, or produce fewer, antibiotics (for
204 brevity, we call these *non-producers*). We first tested the prediction that *producers*
205 are better able to endure the demanding environment (by comparing colony growth
206 rates) than are *non-producers*, and then we directly competed *producers* and *non-*
207 *producers*.

208 We show that *producers* grow relatively more quickly on the *demanding*
209 (*Pseudonocardia*-infused) media despite growing relatively more slowly on the
210 *undemanding* (*Pseudonocardia*-absent) media. We also show that *producers* are the
211 superior competitors on the *demanding* media, while *non-producers* are the superior

212 competitors on the *undemanding* media, as predicted by screening theory. Finally, we
213 confirm the assumption that the *producer* strains are relatively more resistant to
214 antibiotics and the prediction that *Pseudonocardia* Ps2 more strongly suppresses
215 bacterial growth. We emphasise that *producer* and *non-producer* are convenience
216 terms, because the *non-producer* strains that we use can produce antibacterials and
217 exhibit resistance, just to a lesser extent than do the *producers*.

218 Materials & Methods

219 We obtained:

220 (**1**) a set of *Pseudonocardia* Ps1 and Ps2 strains to grow on agar plates.
221 *Pseudonocardia* releases antibacterials and antifungals when grown in culture (Barke
222 *et al.* 2010; Holmes *et al.* 2016; Van Arnam *et al.* 2016);

223 (**2**) a set of 'environmental' *antibiotic-producer* bacterial strains, defined as not
224 having an evolutionary history of growing on the cuticle of *Acromyrmex* ants;

225 (**3**) a set of 'environmental' *non-producer* bacterial strains, defined as not having an
226 evolutionary history of growing on the cuticle of *Acromyrmex* ants; and

227 **(4)** a set of bacterial strains that were isolated in the cuticular microbiomes of
228 *Acromyrmex* workers and based on their taxonomies, should be classed as *non-*
229 *producers*. We asked why these bacteria do not competitively exclude the resident
230 antibiotic-*producers*, since they are presumably resistant to *Pseudonocardia*
231 metabolites but do not pay the cost of producing antibiotics. We use the term
232 'resident' only to indicate that we isolated these strains from *Acromyrmex*; we do not
233 imply that these strains are vertically transmitted like *Pseudonocardia*.

234 For growth media, we used Soya Flour + Mannitol (SFM) agar (20 g soya flour, 20 g
235 mannitol, 20 g agar in 1 L tap water) and lysogeny broth-Lennox (LB-Lennox) media
236 (10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 L deionised water).

237 *Collection and isolation of bacterial strains*

238 (**1**) Nineteen Ps1 and Ps2 strains were isolated from the cuticles of individual workers
239 of the leaf-cutting ant *Acromyrmex echinatior* (Hymenoptera, Formicidae, Attini).
240 The 19 colonies of these workers were collected in Soberania National Park, Panama,
241 between 2001 and 2014, and reared in climate-controlled rooms at the University of
242 Copenhagen at ca. 25 ºC and 70% relative humidity. Worker propleural plates were
243 scraped with a sterile needle under a stereomicroscope and streak-inoculated across
244 LB-Lennox media under sterile conditions. The plates were then incubated at 30 °C
245 (standard culturing temperature) until visible growth of distinctive white 'cotton ball'
246 colonies of *Pseudonocardia* were apparent. These individual colonies were removed
247 under sterile (LAF bench) conditions and inoculated onto fresh plates, a process that
248 was repeated until clean *Pseudonocardia* cultures were isolated. The *Pseudonocardia*
249 isolates were then grown on Mannitol Soya Flour (SFM) media (optimal for
250 actinobacterial growth and spore production), from which spore stock solutions were
251 prepared in 20% glycerol and kept at -20 °C until use. Each isolate was genotyped to
252 Ps1 or Ps2 (Supplementary Information S1). Five strains from each of these two
253 species have previously been genome-sequenced and formally described as different
254 *Pseudonocardia* species by Holmes *et al*. (2016): *P. octospinosus* (Ps1) and *P.*
255 *echinatior* (Ps2).

256 (**2**) The 10 environmental *producer* strains (all *Streptomyces*) were taken from general
257 collections in the Hutchings lab (Tables 1, S2).

258 (**3**) The 10 environmental *non-producer* strains were obtained from the Hutchings lab
259 (2 strains) and from the ESKAPE suite (8 strains) – a set with varying origins (human
260 skin, soil, etc.) associated with hospital-acquired infections and used to test antibiotic
261 resistance or efficacy in clinical/research settings (Tables 1, S2).

262 (**4**) The 10 *Acromyrmex*-resident, *non-producer* strains were isolated from *A.*
263 *echinatior* and *A. octospinosus* ants by culturing from cuticular washes. These strains
264 were 16S-genotyped and where possible assigned to genus by comparison to the
265 NCBI 16S RefSeq database (Supplementary Information S1, Tables 1, S2).

266 Hereafter, for brevity, we refer to the environmental *non-producers* simply as *non-*
267 *producers* and continue to use the full term 'resident *non-producers*' to differentiate
268 them.

269 *Individual growth-rate experiments*

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

272 To create the *Pseudonocardia*-infused media, we grew lawns of the 19
273 *Pseudonocardia* isolates (Table 1), plating 30 µl on 90 mm SFM agar plates. The
274 control plates were inoculated with glycerol only (20%; identical to that used for
275 making solutions from *Pseudonocardia* strains). We incubated these plates at 30 °C
276 for 6 weeks, which ultimately produced lawns from 17 strains that could be included
277 in the experiments (6 Ps1, 11 Ps2). Once each plate was fully (not necessarily
278 uniformly) covered, we flipped the agar to reveal a surface open for colonisation.

279 The 10 environmental *producers* and 10 environmental *non-producers* (Table 1) were
280 inoculated onto the plates. Each plate was inoculated with 10 evenly spaced colonies,
281 with 3 replicates, generating 2 invader types x 17 Ps-media-types x 3 replicates = 102

282 Ps-infused plates and 2 invader types x 3 replicates = 6 control plates, for 1020
283 treatment and 60 control inoculations. Each strain inoculation used 5 µl of solution
284 (approx. 1 x 10^6 cells per ml in 20% glycerol), spotted at evenly spaced positions and
285 without coming into direct contact. All plates were incubated at 30 °C for five days,
286 after which photographs were taken.

287 Images were processed in Fiji software (Schindelin *et al.* 2012; Rueden *et al.* 2017),
288 creating binary negatives (black & white) so automated tools could identify discrete
289 areas of growth (black) and measure growth areas for each invading strain; in the few
290 cases where binary image resolution was insufficient, outlines were added manually
291 before area calculation. 48 *producer*-inoculated and 57 *non-producer*-inoculated
292 treatment measurements were excluded because plate condition had deteriorated to
293 become unscorable or they were contaminated, leaving final sample sizes of 1020-48-
294 57= 915 treatment inoculations and 60 controls.

295 The second growth-rate experiment compared the 10 *Acromyrmex*-resident, *non-*
296 *producer* strains with 9 of the environmental *producer* strains (1 of the 10
297 inoculations failed to grow). All 19 *Pseudonocardia* strains grew sufficiently to be
298 included in this experiment, and each plate was again inoculated with 10 or 9 evenly
299 spaced colonies. Starting sample sizes were therefore 2 invader types x 19 Ps-media-
300 types x 3 replicates = 114 Ps-infused plates and 2 x 3 = 6 control plates, for 1083
301 treatment and 57 control inoculations. 50 *producer* and 20 *non-producer* treatment
302 measurements were excluded for the same reasons as above, leaving final sample
303 sizes of 1083-50-20=1013 treatment and 57 control inoculations, scored as above.

304 *Pairwise competition experiment*

305 We then tested whether *producers* could win in direct competition against *non-*
306 *producers*. We co-inoculated pairs of environmental *producers* and *non-producers* on
307 *Pseudonocardia*-infused media and on control media (prepared as in the individual
308 growth-rate experiment), and we measured the outcome of competition as a win, loss,
309 or draw. The screening model (Scheuring & Yu 2012) predicts that the *producer*
310 strains is more likely to win when growing on *Pseudonocardia*-infused media.

311 To keep the number of tests manageable, we used two combinations of
312 *Pseudonocardia*-infused media and *Streptomyces*: *Pseudonocardia* Ps1 (strain
313 Ae707) + *Streptomyces* S8 and *Pseudonocardia* Ps2 (strain Ae717) + *Streptomyces*
314 S2 (Table 1). These combinations were representative of the results from the growth-
315 rate experiment: the two *Streptomyces* strains grew more slowly than most of the
316 *non-producers* on control media and either near the median growth rate of (S8) or
317 faster than (S2) the *non-producers* on the media infused by these *Pseudonocardia*
318 strains.

319 We competed the two *Streptomyces* strains (S2, S8) against the 10 environmental
320 *non-producer* strains (20 pairings), using *Streptomyces* spore titrations consisting of
321 10^6 spores per ml for each strain in 20% glycerol. The *non-producer* strains were
322 grown overnight in 10 ml LB-Lennox, subcultured with a 1:100 dilution into 10 ml of
323 fresh LB-Lennox, and grown at 37 °C for 3-4 hours, after which OD_{600} was measured,
324 assuming that $OD_{600} = 1$ represented 8×10^9 cells. Similar dilutions of 10^6 cells per
325 ml were made for each *non-producer* strain in 20% glycerol, after which *producer*
326 and *non-producer* dilutions were mixed 1:1. Each pair of *producer* and *non-producer*
327 was co-inoculated as a mixture of 20 µl (10^4 spore-cells of each) on the designated
328 *Pseudonocardia*-infused media (set up as above) with 5 replicates per pairing. We

329 used 150 plates for the S8 experiment (including 100 control plates; 10 replicates per
330 pairing) and 100 plates for the S2 experiment (including 50 control plates; 5 replicates
331 per pairing). Plates were incubated at 30 °C for 5 days and then photographed, after
332 which images were scored with respect to the *producer* as: win (dominant growth),
333 draw (both strains growing with no clear dominant), or loss (little or no visible
334 growth), with reference to images of each strain grown alone on control media to
335 minimise observer bias. One plate's outcome was too ambiguous to score and was
336 omitted. All plates were independently scored by two observers, one using photos,
337 which produced datasets giving the same statistical results. We report the direct
338 observer's scores.

339 *Antibiotic-resistance profiles*

340 Our key assumption is that *producer* strains (here, all *Streptomyces*) are relatively
341 better at resisting antibiotics, because this correlation is what confers competitive
342 superiority on a toxic host surface. We tested this assumption by growing the 10
343 environmental *producer* strains and the 10 environmental *non-producer* strains (Table
344 1, S2) in the presence of 8 different antibiotics, representing a range of chemical
345 classes and modes of action. Antibiotics were added to 1 ml of LB-Lennox medium in
346 a 24-well microtitre plate at 6 different concentrations. The relative concentration
347 range was the same for each antibiotic, although actual concentrations reflected
348 activity (Table S3). *Producers* and *non-producers* were inoculated onto the plates and
349 incubated at 30 °C for 7 days, then photographed (Table S3). Lowest Effective
350 Concentration (LEC, lowest concentration with inhibitory effect) and Minimum
351 Inhibitory Concentration (MIC, lowest concentration with no growth) scores were
352 assigned on a Likert scale of 1–6, where 1 was no resistance and 6 was resistance
353 above the concentrations tested (adapted from generalised MIC methods; reviewed by

354 Balouiri *et al.* 2016). The Likert scale is ordinal, so we analysed using a non-
355 parametric test.

356 *Justification of experimental-design choices*

357 Petri-dish experiments are analogous to greenhouse experiments in plant ecology in
358 that they trade off realism for improved inference of cause and effect, but microbial
359 experiments face the additional challenge of having to use macroscopic elements to
360 mimic microscopic interactions. For instance, we stage competition between pairs of
361 strains at the macroscopic scale because it is likely to be the more realistic
362 representation of competition at the microscopic scale, since interactions are local and
363 colonising spores from the air arrive slowly and settle randomly. These considerations
364 cause the instantaneous probability of interaction between strains to be low, and the
365 losing strain to be excluded before a new invader arrives in the same position,
366 rendering competition effectively pairwise in real microbiomes. Pairwise competition
367 is also more conservative in that it should maximise the competitiveness of each strain
368 competed against *Streptomyces*. If multiple *non-producer* strains were mixed and
369 inoculated together, they would also compete against each other (i.e. 'an all-out
370 melee'), diminishing the competitiveness of any given strain against *Streptomyces*.

371 We also conservatively chose not to replenish nutrients after growing *Pseudonocardia*
372 to make the demanding plates, because increased food resources favour *producers*,
373 since antibiotic synthesis and resistance are costly (Scheuring & Yu 2012). Finally,
374 we included ESKAPE strains as *non-producers* because they grow well in lab
375 conditions, thus removing an artefactual cause of competitive inferiority.

376 *Statistical analyses*

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

386 **Results**

387 *Individual growth-rate experiments*. – As predicted by the screening model, the
388 environmental *non-producers* grew more quickly on the undemanding control media
389 while *producers* grew more quickly on the demanding *Pseudonocardia*-infused
390 media, producing a highly significant statistical interaction effect (Fig. 1A). There
391 was also a significant main effect of *Pseudonocardia* genotype, with both *non-*
392 *producers* and *producers* exhibiting a lower growth rate on Ps2-infused media.

393 The resident *non-producers* isolated from cuticular microbiomes had significantly
394 slower growth rates overall, even on the non-toxic control media (Fig. 1B). This
395 explains why they are unable to outcompete *producer* strains but raises the question
396 of why these *non-producer* species persist at all. We return to this in the Discussion.

397 *Pairwise competition experiment*. – When grown on the demanding *Pseudonocardia*-
398 infused media, the *producer* strains were much more likely to win in direct

399 competition against the *non-producer* strains (Fig. 2). Draws were more common on
400 the Ps1-infused plates.

401 *Antibiotic-resistance profiles*. – The screening model assumes that the *producer*
402 strains are more resistant to antibiotics than are the *non-producer* strains. We
403 confirmed this assumption; the Lowest Effective Concentration (LEC) and the
404 Minimum Inhibitory Concentration (MIC) scores were both greater for the *producer*
405 strains (Fig. 3). We also demonstrated that the *resident non-producer* strains had high
406 levels of antibiotic resistance (Fig. 3), as expected, given that they were isolated from
407 ant cuticles (Fig. 1B).

408 Discussion

409 We used *in vitro* experiments to test key predictions and assumptions of a screening
410 model (Scheuring & Yu 2012) that explains how leaf-cutting ants could use a
411 vertically transmitted symbiont, *Pseudonocardia*, to selectively recruit additional
412 antibiotic-producing bacterial strains out of the large pool of potential bacterial
413 symbionts present in the environment.

414 We showed that *producer* strains grow relatively faster than do *non-producer* strains
415 on *Pseudonocardia*-infused toxic media (Fig. 1A). Since the *non-producers* have
416 faster growth on the non-toxic control plates, we attribute the *producers'* relatively
417 faster growth on toxic media to greater inhibition of *non-producers* by
418 *Pseudonocardia* metabolites, consistent with the large number of antibacterials
419 documented in *Pseudonocardia*'s genome (Holmes *et al.* 2016). This interpretation is
420 supported by the experiment showing that the *producer* strains are more resistant to a
421 wide range of antibiotics than are the *non-producer* strains (Fig. 3) and is consistent
422 with the result that *Pseudonocardia* Ps2 is a stronger suppressor of bacterial growth

423 (Fig. 1A), since Ps2-hosting *Acromyrmex* workers host lower-diversity (and more
424 Ps2-dominated) cuticular microbiomes (Andersen *et al*. 2013).

425 Most importantly, the pairwise competition experiment confirmed that growing on
426 *Pseudonocardia*-infused media renders *producer* strains competitively superior (i.e.
427 they usually win) or at least resistant to exclusion (they more often draw than lose,
428 especially on the less-toxic Ps1-infused media) (Fig. 2). This experiment mimics the
429 life-history stage of *Acromyrmex* workers after their full-body *Pseudonocardia* bloom
430 has retreated to the propleural plates (and possibly into the exocrine glands) and they
431 have begun to forage outside the nest, where they are exposed to bacterial colonists
432 (Poulsen *et al.* 2005; Andersen *et al.* 2013; Marsh *et al.* 2014; Andersen *et al.* 2015).

433 Taken together, these results show how a vertically transmitted, antibiotic-producing
434 symbiont like *Pseudonocardia* can be used by the ant host to create a demanding
435 environment that biases competition in favour of antibiotic-producers, favouring their
436 successful colonisation. In other words, vertical transmission of one mutualist can
437 enable the successful horizontal transmission of other mutualists. Vertical
438 transmission and horizontal transmission are therefore not competing explanations,
439 consistent with the two transmission modes being found together in other symbioses
440 (Ebert 2013; Bourguignon *et al.* 2018; da Costa & Poulsen 2018; Ivens *et al.* 2018).
441 Under other circumstances, mutualistic recruitment via screening may also be
442 achievable without a vertically transmitted symbiont; Duarte *et al*. (2018) have
443 argued that a host might be able to create a suitably demanding environment by
444 evolving the right set of chemical exudates.

445 We emphasise that the outcomes of the pairwise competition experiment are the
446 *emergent* outcomes of a complex interplay of mechanisms and stochasticity, including
447 the production, export, diffusion, and decay of a diverse set of antibacterials being

448 blocked, decomposed, neutralised, sequestered, and re-exported by a diverse set of
449 resistance mechanisms, spatially (and possibly temporally) heterogeneous resource
450 supply by the host, and community bistability via space taking caused by stochastic
451 orders of arrival, playing out across innumerable local interactions. A mechanistic
452 model incorporating these complications (Boza *et al*. 2018) still results in the same
453 emergent outcomes as in the original phenomenological model (Scheuring & Yu
454 2012). Based on this modelling, our best (and still simplified) guess of what is going
455 is that the *Streptomyces* strains are relatively more efficient than the *non-producer*
456 strains at resisting toxic *Pseudonocardia* antibacterials (Fig. 3). This efficiency
457 advantage lets *Streptomyces* devote a larger proportion of available resources toward
458 colony growth (Fig. 1A) and toward production of their own antibacterials (e.g.
459 Schoenian *et al*. 2011), the combined effect of which lets *Streptomyces* capture open
460 space more rapidly than its competitors. This scenario does not require the screened-
461 out bacteria to have zero ability to synthesise and resist antibacterials, just less ability.

462 We note that the ant can employ *Pseudonocardia* as a screening mechanism
463 regardless of whether *Pseudonocardia*'s fitness is aligned with the ant, because the
464 ant controls where and when host resources are provided, thus governing
465 *Pseudonocardia*'s spatial distribution and biomass. We also note that since virgin
466 *Acromyrmex* queens are inoculated upon hatching by nurse workers that maintain
467 *Pseudonocardia* monocultures, the acquisition of other symbionts by foraging
468 workers should not disrupt verticality in the transmission of *Pseudonocardia*.

469 Acromyrmex-*resident non-producers*. – We also ran a growth-rate experiment with a
470 set of *resident non-producer* strains that had been isolated from *Acromyrmex* ant
471 cuticles (Fig. 1B) and found that they generally had low growth rates, making it
472 unlikely that they can competitively exclude *producers*. It seems surprising that we

473 found these *non-producer* strains at all, but we note that the fact that we could isolate
474 them does not imply that they are abundant. Resident *non-producer* taxa are only
475 found on some (not all) field-sampled *Acromyrmex* workers (Andersen *et al.* 2013; T.
476 Innocent *et al*., *unpubl. data*) and generally below 1% as a proportion of amplicon
477 read number. They may only be transient environmental contaminants, since many
478 were isolated via whole-ant washes, or they might be persisting via non-equilibrium
479 dynamics. In simulation models (Boza et al. 2018), we have found that if bacterial
480 competition is limited to nearest neighbours and antibiotics slowly diffuse across the
481 ant surface, even non-antibiotic-resistant strains can sometimes persist for a long time.

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

493 Comparable studies to ours using other ant species will be needed to test the
494 generalisability of screening to other attine ant lineages that maintain actinobacterial
495 cuticular microbiomes, including species that appear to have cuticular microbiota with
496 abundant actinomycete genera other than *Pseudonocardia* (Sen *et al.* 2009; Andersen
497 *et al.* 2013; Meirelles *et al.* 2014). Our results suggest that cuticular microbiomes and

host resource provisioning can be simulated on agar plates adequately enough for experimentation, providing a tractable method with which to test microbiome assembly mechanisms, eventually leading to *in vivo* experiments.

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 microbiomes, 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; 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 game theory 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 mechanisms to foment and bias competition by modifying resource and toxicity levels to encourage the assembly of mutualistic microbial communities (see Foster *et al.* 2017; Duarte *et al.* 2018 for similar perspectives).

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761 # Figure legends

762 **Figure 1**. Individual growth-rate experiments. Bacterial colony sizes after 5 days at 30 ºC,
763 with the boxplots indicating medians ± one quartile. The white section shows growth rates
764 on control media, and the grey section shows growth rates on the *Pseudonocardia*-infused
765 media. Coral boxes represent *non-producer* strains, and blue boxes represent *producer*
766 strains. For analysis, a linear mixed-effects model, including *Pseudonocardia* strain
767 (`Ps.strain`, 17 groups) and inoculated bacterial species (`Inv.strain`, 20 groups) as
768 random intercepts, was used to test for interaction and main effects of growth media
769 (`Control` vs. `Ps1.infused` vs. `Ps2.infused`) and antibiotic production
770 (`Non.producers` vs. `Streptomyces`): (*`lme4::lmer`*`(Growth.score ~ Media *`
771 `Antibiotic.production + (1|Ps.strain/Plate) + (1|Inv.strain))`. **A.**
772 Environmental strains. There was a highly significant interaction effect, such that
773 environmental non-producer strains grow more rapidly on control media, while
774 *Streptomyces* grow more rapidly on *Pseudonocardia*-infused media (n = 975, χ^2 = 45.86, df =
775 2, p < 0.0001). There was also a highly significant main effect of *Pseudonocardia* genotype,
776 with growth being lower on Ps2-infused media (n = 915, χ^2 = 24.55, df = 1, p < 0.0001,
777 control-media data omitted for this analysis). One non-producer strain (*Staphylococcus*
778 *epidermidis*) grew more rapidly than all other strains (open triangle points), demonstrating
779 the need to control for correlated residuals by strain. The y-axis has been truncated at 2.5
780 for clarity (the full figure is in Supplementary Information). **B.** *Acromyrmex*-resident, *non-*
781 *producer* strains. There was no interaction effect (χ^2 = 2.64, df = 2, p = 0.27), but both main
782 effects were highly significant. The *non-producers* grow more slowly on all media types (χ^2 =
783 20.96, df = 1, p < 0.0001), and bacterial growth was generally slower on Ps2-infused media
784 (χ^2 = 21.43, df = 1, p < 0.0001, control-media data omitted).

785

786 **Figure 2.** Pairwise competition experiment, scoring whether or not *producers* prevail. **A**.
787 Representative images of agar plates at five days post-inoculation showing examples of the
788 three competitive outcomes: Win (S2 vs. St3 on Ps2 media), Draw (S2 vs. St3 on control
789 media), and Loss (S8 vs. St3 on control media; strain details in Table 1). **B**. Barcharts of
790 competitive outcomes for the two *Streptomyces producer* strains (S8 and S2; Table 1), each
791 pairwise co-inoculated with ten non-antibiotic-producer strains on *Pseudonocardia*-infused
792 media and on control media. For analysis, draw outcomes were omitted, and a general
793 linear mixed-effects model, including non-antibiotic-producer strain as a random intercept
794 (10 groups), was used to test for an effect of the `medium` term (`Control` vs. `Ps1/2-`
795 `infused`) on competitive outcome (`Win` vs. `Loss`) (`(lme4::glmer(outcome ~`
796 `medium + (1|non.producer.strain), family = binomial))`. Significance was
797 estimated using term deletion. In both experiments, *producer* strains (*Streptomyces*) were
798 more likely to be competitively superior when grown on the *Pseudonocardia*-infused media
799 (Left: n = 129, χ^2 = 103.6, df = 1, p < 0.0001; Right: n = 94, χ^2 = 87.9, df = 1, p < 0.0001).

800

801 **Figure 3.** Antibiotic resistance profiles for *producer*, *non-producer*, and *resident non-*
802 *producer* strains (Table 1). Boxplots indicate medians (notches) ± one quartile. For analysis,
803 we calculated each strain's mean growth score across the eight tested antibiotics (reducing
804 from n = 155 to n = 20; details in S3). *Producers* showed higher levels of resistance than did
805 *non-producers* for both measurements: Wilcoxon two-sided test (`wilcox.test`), W =
806 94.5, p = 0.0017 for LEC (**A**) and W = 80, p = 0.0253 for MIC (**B**), after correction for two tests
807 (`p.adjust(method="fdr")`). *Producers* and *Resident Non-producers* showed no
808 difference in resistance levels (p = 0.44 and 0.25). The 'rabbit ears' in **B** indicate that the
809 medians are also the highest values.

810

811 # Tables, Figures & Boxes

812 **Table 1.** The bacterial strains used in our experiments: the 19 *Pseudonocardia* strains isolated from
813 *Acromyrmex echinatior* (columns 1 and 2); the 10 environmental *antibiotic-producer* strains (column 3);
814 the 10 environmental *antibiotic-non-producer* strains (column 4); and the 10 *Acromyrmex-resident,*
815 *antibiotic- non-producer* strains (column 5). Details for all strains not isolated from *Acromyrmex* in Table
816 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	-	-	-

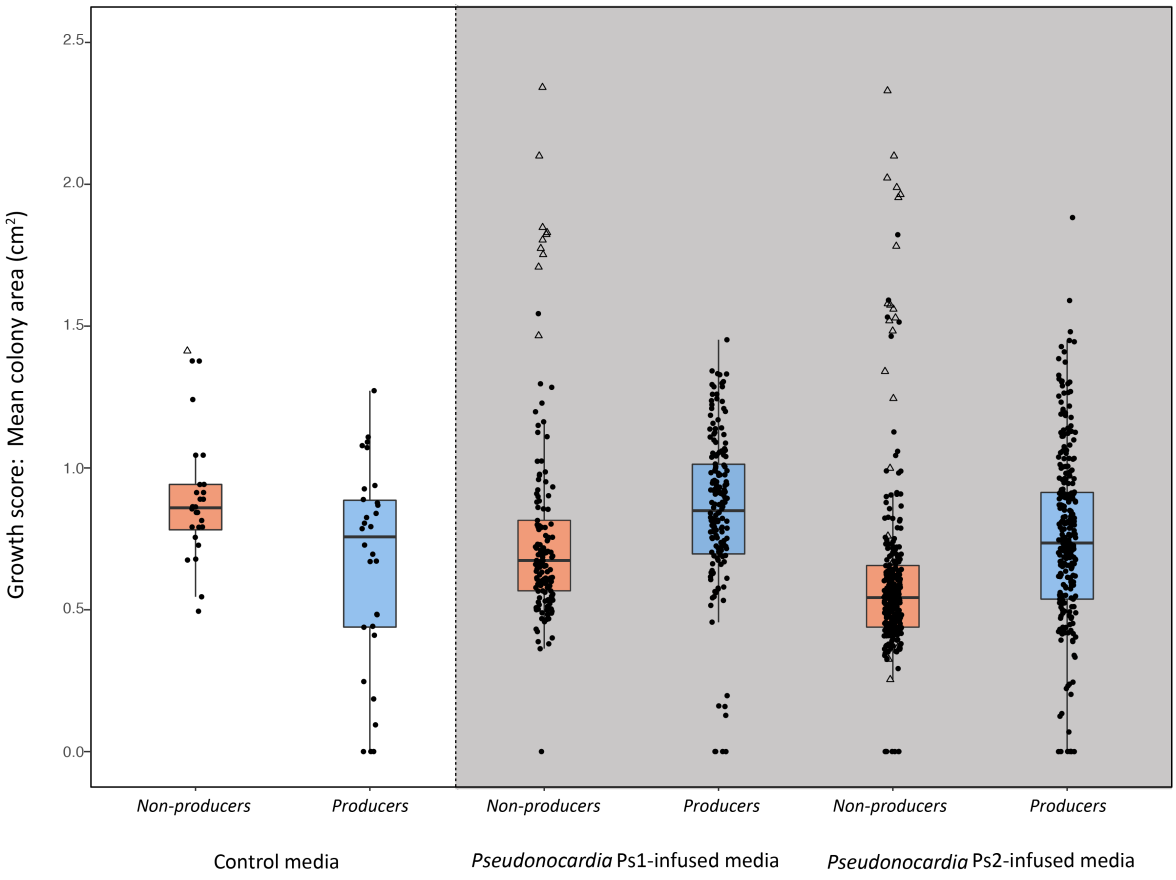
* *Pseudonocardia* strains that have been genome-sequenced (Holmes *et al.* 2016)
† *Pseudonocardia* strains that were only used in the growth-rate experiment with *Acromyrmex*-resident *non-producers*.

817

818

819 **Figure 1.**

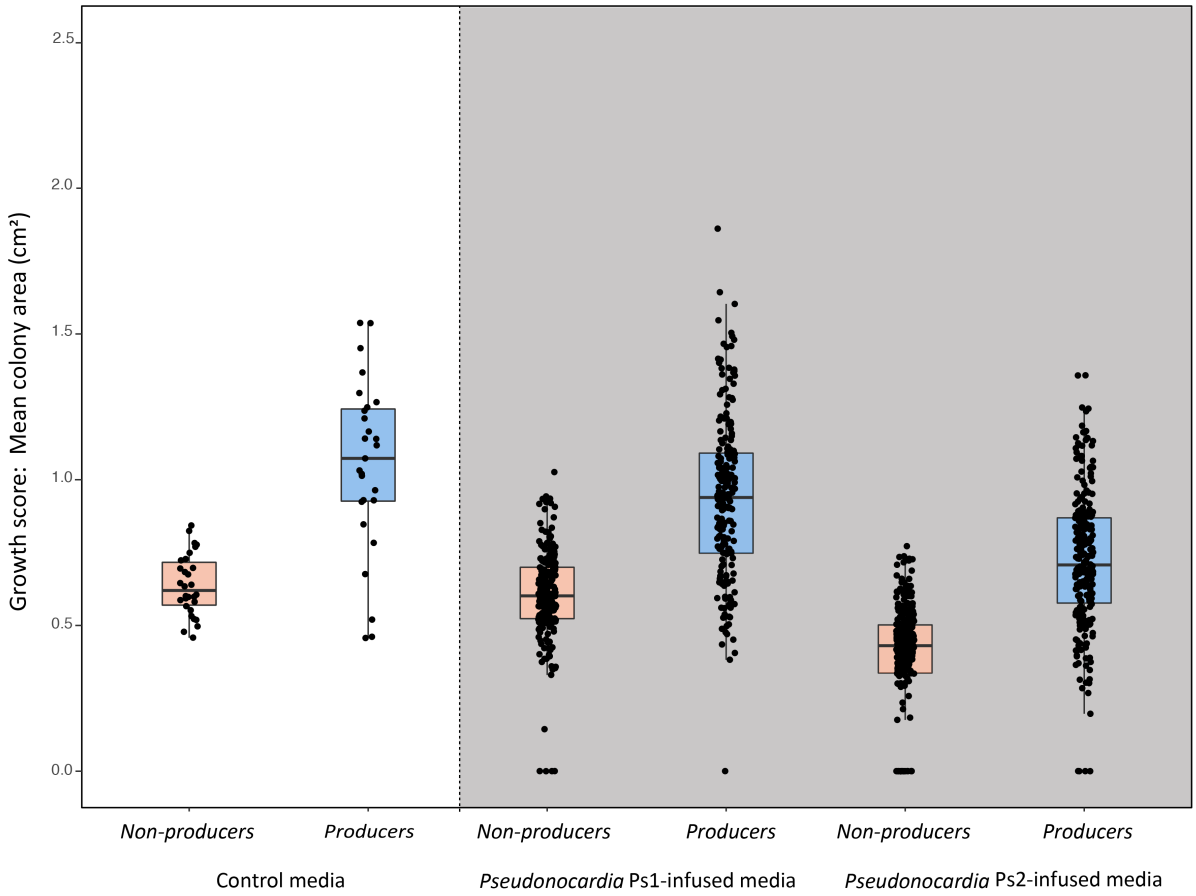
A. Growth rates of environmental *non-producers* vs. *producers*



820

821

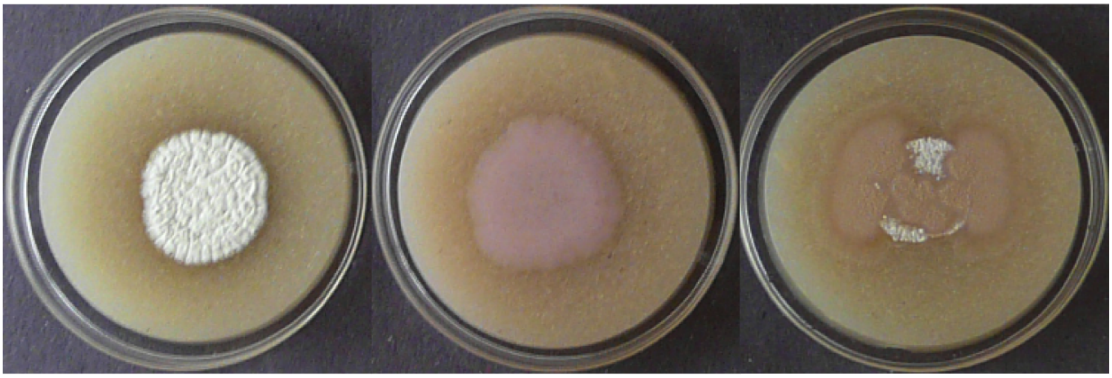
B. Growth rates of *Acromyrmex* resident *non-producers* vs. *producers*



822

823 **Figure 1**. Individual growth-rate experiments. Bacterial colony sizes after 5 days at 30 ºC,
824 with the boxplots indicating medians ± one quartile. The white section shows growth rates
825 on control media, and the grey section shows growth rates on the *Pseudonocardia*-infused
826 media. Coral boxes represent *non-producer* strains, and blue boxes represent *producer*
827 strains. For analysis, a linear mixed-effects model, including *Pseudonocardia* strain
828 (`Ps.strain`, 17 groups) and inoculated bacterial species (`Inv.strain`, 20 groups) as
829 random intercepts, was used to test for interaction and main effects of growth media
830 (`Control` vs. `Ps1.infused` vs. `Ps2.infused`) and antibiotic production
831 (`Non.producers` vs. `Streptomyces`): (*`lme4::lmer`*`(Growth.score ~ Media *`
832 `Antibiotic.production + (1|Ps.strain/Plate) + (1|Inv.strain))`.
833 **A.** Environmental strains. There was a highly significant interaction effect, such that
834 environmental non-producer strains grow more rapidly on control media, while
835 *Streptomyces* grow more rapidly on *Pseudonocardia*-infused media (n = 975, χ^2 = 45.86, df =
836 2, p < 0.0001). There was also a highly significant main effect of *Pseudonocardia* genotype,
837 with growth being lower on Ps2-infused media (n = 915, χ^2 = 24.55, df = 1, p < 0.0001,
838 control-media data omitted for this analysis). One non-producer strain (*Staphylococcus*
839 *epidermidis*) grew more rapidly than all other strains (open triangle points), demonstrating
840 the need to control for correlated residuals by strain. The y-axis has been truncated at 2.5
841 for clarity (the full figure is in Supplementary Information). **B.** *Acromyrmex*-resident, *non-*
842 *producer* strains. There was no interaction effect (χ^2 = 2.64, df = 2, p = 0.27), but both main
843 effects were highly significant. The *non-producers* grow more slowly on all media types (χ^2 =
844 20.96, df = 1, p < 0.0001), and bacterial growth was generally slower on Ps2-infused media
845 (χ^2 = 21.43, df = 1, p < 0.0001, control-media data omitted).

Figure 2.



Streptomyces win *Streptomyces* loss Draw

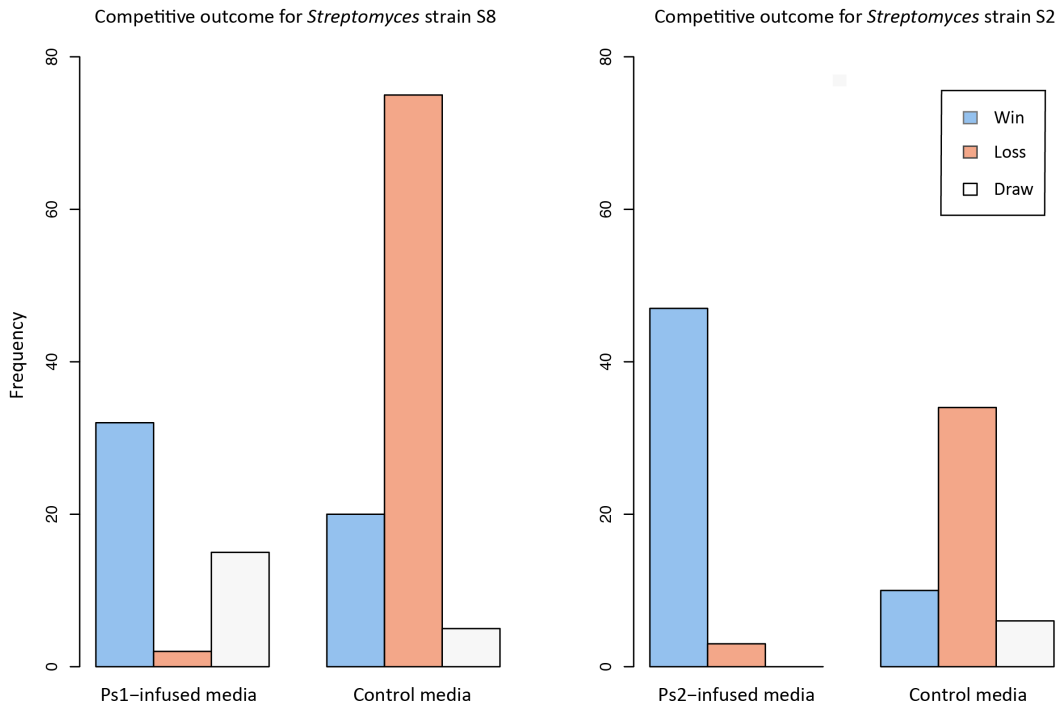
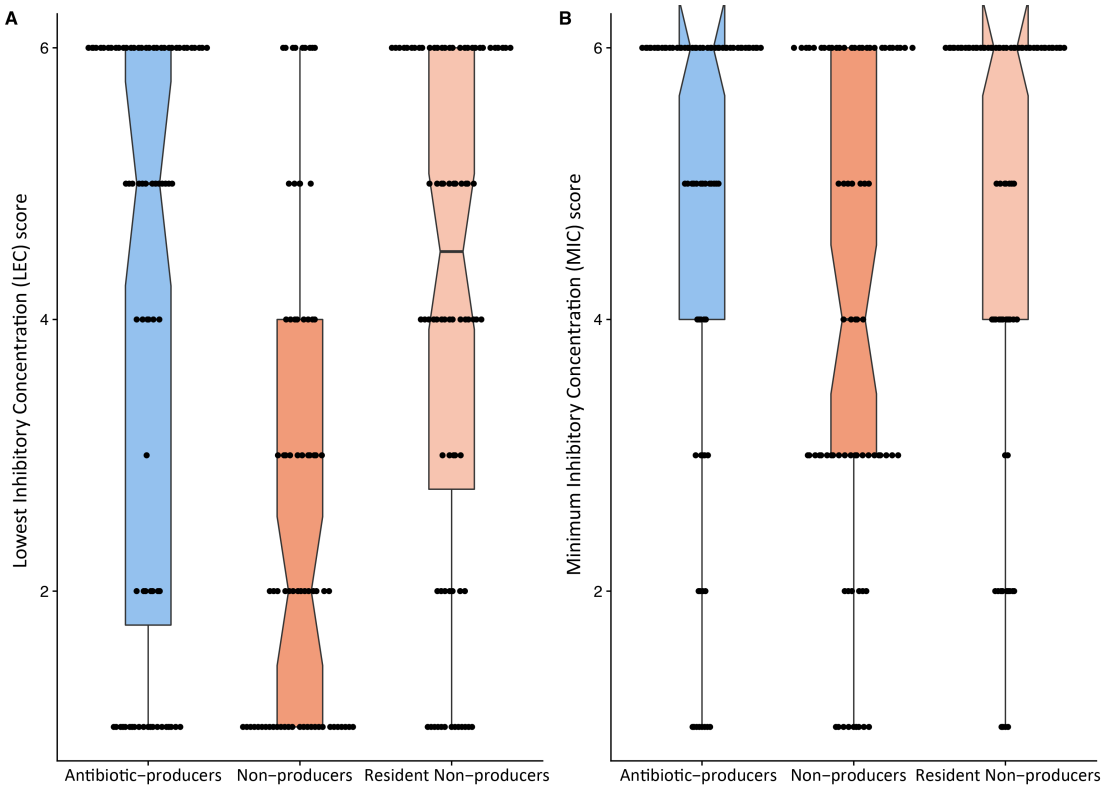


Figure 2. Pairwise competition experiment, scoring whether or not *producers* prevail. **A**. Representative images of agar plates at five days post-inoculation showing examples of the three competitive outcomes: Win (S2 vs. St3 on Ps2 media), Draw (S2 vs. St3 on control media), and Loss (S8 vs. St3 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).

862 **Figure 3.**



863

864 **Figure 3.** Antibiotic resistance profiles for *producer*, *non-producer*, and *resident non-producer*
865 strains (Table 1). Boxplots indicate medians (notches) ± one quartile. For analysis, we
866 calculated each strain's mean growth score across the eight tested antibiotics (reducing from
867 n = 155 to n = 20; details in S3). *Producers* showed higher levels of resistance than did *non-*
868 *producers* for both measurements: Wilcoxon two-sided test (`wilcox.test`), W = 94.5, p =
869 0.0017 for LEC (**A**) and W = 80, p = 0.0253 for MIC (**B**), after correction for two tests
870 (`p.adjust(method="fdr")`). *Producers* and *Resident Non-producers* showed no
871 difference in resistance levels (p = 0.44 and 0.25). The 'rabbit ears' in **B** indicate that the
872 medians are also the highest values.

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

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28 **S1. Genotyping of *Pseudonocardia octospinosus* (Ps1) and *P. echinatior* (Ps2)**
29 **strains, and *Acromyrmex* resident *non-producer* strains**

30 We confirmed the *Pseudonocardia* genotype of the 19 strains – *P. octospinosus* (Ps1)
31 or *P. echinatior* (Ps2) – using material from a parallel study (T. Innocent *et al*., in
32 prep) where DNA extractions were carried out using the Qiagen Blood & Tissue
33 DNA kit (following the manufacturers' protocol, with an additional step of vortexing
34 each sample for 30 seconds with 0.1 mm glass beads at the first stage; each sample
35 was re-eluted in 100µl AE elution buffer).

36 We amplified part of the elongation factor EF-Tu gene using the *Pseudonocardia*-
37 specific primers 52F and 920R (Ludwig *et al.* 1993; Poulsen *et al.* 2005). We ran
38 20 μL PCR reactions using the VWR 2x PCR Mix (VWR, USA) and with the
39 following conditions: 96°C for 2.5 minutes; 40 cycles of 94°C for 45s, 56°C for 50s,
40 72°C for 2 minutes; and a final extension period of 10 minutes at 72°C. Following
41 PCR amplification, confirmed by observing a single band on 2% agarose gel, DNA
42 was purified using a MSB Spin PCRapace kit (STRATEC Molecular GmbH). All
43 samples were prepared for sequencing using a Mix2Seq kit (Eurofins genomics) and
44 sent to and directly sequenced at MWG Eurofins (MWG, Germany).

45 We aligned all sequences, and compared to existing Ps1 and Ps2 consensus sequences
46 (Ps1, [GenBank: DQ098127]; Ps2, [GenBank: DQ098133]), using MUSCLE
47 implemented in Geneious R7.1, to assign each strain to one or other species.

48 The 10 *Acromyrmex*-resident, *non-producer* strains were 16S-genotyped, with strains
49 first grown on agar plates, added to 100 µl 50% DMSO and heated 65°C for 20
50 minutes. We then amplified 16S rDNA from this material using the primers 28F (5′-
51 GAGTTTGATCNTGGCTCAG-3′) and 519R (5′- GWNTTACNGCGGCKGCTG-3′).

52 We ran 50 μL PCR reactions using BIOTAQ™ Red DNA Polymerase Mix (Bioline)
53 and with the following conditions: 95°C for 1 minute; 30 cycles of 95°C for 15s,
54 55°C for 15 s, 72°C for 15s; and a final extension period of 2 minutes at 72°C.
55 Following PCR amplification, confirmed by observing a single band on 1% agarose
56 gel, DNA was purified using a QIAquick Gel Extraction kit (QIAGEN). All samples
57 were prepared for sequencing using a Mix2Seq kit (Eurofins genomics) and sent to
58 and directly sequenced at MWG Eurofins (MWG, Germany).

59

60 **S2. Source information for all experimental strains**

Strain	**Source, Reference**.
S. coelicolor M1146	John Innes Centre, Norwich, NR4 7UH, UK, Gomez-Escribano & Bibb 2011
S. lividans	John Innes Centre, Norwich, NR4 7UH, UK, Hopwood *et al.* 1983
S. coelicolor M145	John Innes Centre, Norwich, NR4 7UH, UK, Bentley *et al.* 2002
S. scabies 87-22	Bignell *et al.* 2010
S. venezuelae NRRL B-65442	USDA ARS Culture Collection. https://nrrl.ncaur.usda.gov/cgi-bin/usda Strain no. B-65442.
S. Ae150A-B1	*A. echinatior* derived
S. Ae356-S1	*A. echinatior* derived
S. formicae KY5	Seipke *et al.* 2013; Holmes *et al.* 2018
S. S4	Barke *et al.* 2010; Seipke *et al.* 2011
S. S4 Δ*antA::apr*	Seipke *et al.* 2014
Escherichia coli (ESKAPE strain)	ATCC® 11775™
Lysobacter antibioticus (ESKAPE strain)	Handelsman Lab, Small World Initiative, University of Wisconsin-Madison, USA
Bacillus subtilis (ESKAPE strain)	Handelsman Lab, Small World Initiative, University of Wisconsin-Madison, USA

Pseudomonas putida (ESKAPE strain)	Handelsman Lab, Small World Initiative, University of Wisconsin-Madison, USA
Erwinia caratova (ESKAPE strain)	Handelsman Lab, Small World Initiative, University of Wisconsin-Madison, USA
Enterobacter aerogenes (ESKAPE strain)	ATCC® 51697™
Acinetobacter baylyi (ESKAPE strain)	ATCC® 33305™
Staphylococcus epidermidis (ESKAPE strain)	ATCC® 14990™
Micrococcus luteus (ESKAPE strain)	ATCC® 4698™
Serratia KY15 (ESKAPE strain)	Seipke *et al.* 2013
Sr1. Ochrobactrum sp.	This study
Sr2. Erwinia sp.	This study
Sr3. Acinetobacter *sp.*	This study
Sr4. Sphingobacterium sp.	This study
Sr5. Acinetobacter *sp.*	This study
Sr6. Luteibacter sp.	This study
Sr7. Flavobacterium sp.	This study
Sr8. Brevundimona sp.	This study
Sr9. Acinetobacter sp.	This study
Sr10. Brachybacterium sp.	This study

S3. Detailed methods for antibiotic MIC tests

Antibiotic-producer strains: From spore titrations of *Streptomyces*, 1 x 10^6 spores of each strain were plated onto SFM media with each of the eight antibiotics at six concentrations (Table S3).

Environmental non-producer and Acromyrmex-*resident non-producer strains*: These were grown over 1-2 nights in 10 ml Lennox; sub-cultured with a 1:100 dilution into a fresh 10 ml Lennox; grown again at 37 °C for 3-4 hours (for slower growers the

70 starter culture was used); and OD600 was measured, where OD600 = 1 was assumed
71 to represent 8×10^9 cells. 1 x 10^6 cells of each non-producer strain were then plated
72 onto Lennox media with each of the eight antibiotics at six concentrations (see Table
73 S3).

74 Subsequently, all plates were incubated at 30 °C for 7 days and then photographed;
75 our scoring was based on these images. We examined the set of 6 plates for each
76 strain (10 producers and 10 non-producers) against each of the eight antibiotics (i.e. 6
77 x 8 plates per strain tested) for signs of growth (indicating resistance in the strain).
78 We scored the minimal inhibitory concentration (MIC, the lowest concentration with
79 no growth) and lowest effective concentration (LEC, the lowest concentration with an
80 inhibitory effect) for each pairing of strain x antibiotic (see S4).

81 We then assigned each strain two scores: one based on the antibiotic concentration at
82 which the MIC was reached, and one based on the LEC, on a scale from 1 (if the MIC
83 or LEC was demonstrated at the lowest concentration of antibiotics above 0) to 5 (if
84 the MIC or LEC occurred at the highest of the six concentrations used); we gave a
85 score of 6 if the MIC or LEC was higher than all concentrations of antibiotic used.

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95 ***Table S3. Antibiotic concentrations for MIC tests***

Antibiotic	**Concentrations Used**	**Target**	**Compound type**
Chloramphenicol	0, 2.5, 5, 10, 25, 50 µg/ml	Translation inhibitor	Synthetic
Rifampicin	0, 0.5, 1, 2, 5, 10 µg/ml	Translation inhibitor	Polyketide
Streptomycin	0, 5, 10, 20, 50, 100 µg/ml	Translation inhibitor	Aminoglycoside
Vancomycin	0, 1, 2, 4, 10, 20 µg/ml	Cell wall synthesis inhibitor	Glycopeptide
Phosphomycin	0, 5, 10, 20, 50, 100 µg/ml	Cell wall synthesis inhibitor	Small molecule
Nalidixic Acid	0, 2.5, 5, 10, 25, 50 µg/ml	DNA gyrase inhibitor	Synthetic quinolone
Apramycin	0, 5, 10, 20, 50, 100 µg/ml	Translation inhibitor	Aminoglycoside
Ampicillin	0, 10, 20, 40, 100, 200 µg/ml	Cell wall synthesis inhibitor	ß lactam

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99 **S4.** R markdown-format scripts, input datafiles, and html output files for the analyses

100 in Figures 1, 2, and 3 are provided as a single R project folder and at

101 https://github.com/dougwyu/screening_Innocent_et_al.

102

103

104 **S5. A human example of a successful screening mechanism**

105 Any automobile-breakdown insurance company is faced with a hidden information

106 problem. Potential clients differ in the probability that their cars will need rescue.

107 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.

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