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Interspecies Social Spreading: Interaction between two sessile soil bacteria leads to emergence of surface motility

Running title: Interspecies social spreading as an emergent trait

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1 ## Abstract

2 Bacteria often live in complex communities in which they interact with other organisms.
3 Consideration of the social environment of bacteria can reveal emergent traits and behaviors
4 that would be overlooked by studying bacteria in isolation. Here we characterize a social trait
5 which emerges upon interaction between the distantly-related soil bacteria *Pseudomonas*
6 *fluorescens* Pf0-1 and *Pedobacter* sp. V48. On hard agar, which is not permissive for motility of
7 the mono-culture of either species, co-culture reveals an emergent phenotype we term
8 'interspecies social spreading,' where the mixed colony spreads across the hard surface. We
9 show that initiation of social spreading requires close association between the two species of
10 bacteria. Both species remain associated throughout the spreading colony, with reproducible
11 and non-homogenous patterns of distribution. The nutritional environment influences social
12 spreading; no social behavior is observed under high nutrient conditions, but low nutrient
13 conditions are insufficient to promote social spreading without high salt concentrations. This
14 simple two-species consortium is a tractable model system that will facilitate mechanistic
15 investigations of interspecies interactions and provide insight into emergent properties of
16 interacting species. These studies will contribute to the broader knowledge of how bacterial
17 interactions influence the functions of communities they inhabit.

18 ## Importance

19 The wealth of studies on microbial communities has revealed the complexity and
20 dynamics of the composition of communities in many ecological settings. Fewer studies probe
21 the functional interactions of the community members. Function of the community as a whole
22 may not be fully revealed by characterizing the individuals. In our two-species model
23 community, we find an emergent trait resulting from the interaction of the soil bacteria

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24 *Pseudomonas fluorescens* Pf0-1 and *Pedobacter* sp. V48. Observation of emergent traits
25 suggests there may be many functions of a community that are not predicted based on *a priori*
26 knowledge of the community members. These types of studies will provide a more holistic
27 understanding of microbial communities, allowing us to connect information about community
28 composition with behaviors determined by interspecific interactions. These studies increase our
29 ability to understand communities, such as the soil microbiome, plant-root microbiome, and
30 human gut microbiome, with the final goal of being able to manipulate and rationally improve
31 these communities.

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32 ## Introduction

33 Within soils live a plethora of microbial species that form complex communities
34 responsible for important ecological functions, such as nutrient cycling and plant health. Omics
35 approaches have given us a wealth of information on the composition, diversity, metabolic
36 potential, and ecology of plant- and soil-associated microbial communities (1, 2). However, to
37 get a complete understanding of microbial functions and interactions within these environments,
38 we must look at every layer, from the full community *in vivo* to the individual microbe *in vitro* (3).
39 Historically, research has focused on the study of single species in pure culture, but bacteria are
40 social organisms. Thus, the study of the mechanisms and consequences of multi-species
41 interactions is necessary for us to understand the function of microbial communities as a whole.
42 Investigating entire soil communities *in situ* presents considerable challenges because of
43 fluctuating soil conditions and the wide range of relevant scales, ranging from particulate to
44 ecological levels (2). Reducing the microbial community to pair-wise interactions or small
45 consortia allows for a detailed mechanistic study. This reduction is also an essential link
46 between studying isolated microbes in the laboratory and understanding the collective activities
47 of natural microbial communities (4).

48 Recent work has considered the social environment of bacteria, investigating altered
49 behaviors and production of secondary metabolites when co-cultured with other organisms.
50 Some bacteria exhibit emergent behaviors when presented with other species, likely the result
51 of induction of genes that are not expressed in pure culture. For example, some *Pseudomonas*
52 *fluorescens* strains produce an antifungal compound during interactions with other species (5–
53 9). The co-culture of different actinomycete species results in the production of secondary
54 metabolites, changes in pigment, and sporulation (10–12). The presence of *E. coli* or
55 *Pseudomonas* species effects sporulation and biofilm formation in *Bacillus subtilis* (13, 14). One

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56 subset of social interactions are those which alter the motility behaviors and capabilities of other
57 species. For example, physical association with *Saccharomyces cerevisiae* results in
58 *Streptomyces venezuelae* consuming the yeast and triggers 'exploratory growth' of the bacteria
59 (15). This exploration is not observed when *S. venezuelae* is grown in mono-culture, under the
60 same environmental conditions. In another example, *B. subtilis* moves away from a
61 *Streptomyces* competitor across a solid surface, but does not do so in isolation (16, 17). Other
62 behaviors appear less competitive, where a motile species will travel with a non-motile species
63 that can degrade antibiotics, allowing the consortium to colonize hostile environments (18, 19).
64 *Xanthomonas perforans* can even change the behavior of *Paenibacillus vortex*, producing a
65 signal that induces *P. vortex* to swarm towards it so it can hitchhike (20).

66 *Pseudomonas fluorescens* Pf0-1 and *Pedobacter* sp. V48 are known to interact though
67 diffusible and volatile signals, which induce production of an antifungal compound by *P.*
68 *fluorescens* (6–8). Previous studies with *Pedobacter* and a strain closely-related to *P.*
69 *fluorescens* Pf0-1 (AD21) found that the mixture of the strains showed reciprocal gene
70 expression changes and antagonistic behavior toward the plant pathogen *Rhizoctonia solani* (5,
71 9). The initial study noted expansion of the mixed strains beyond the initial area of inoculation
72 (5), but the phenotype was not characterized, and has not been the focus of any further studies.
73 We investigated this observation using a new assay. Instead of culturing *P. fluorescens* Pf0-1
74 and *Pedobacter* without contact, as was done in the antagonism assays (6), we mix them
75 together. We hypothesized that, while antibiotic production can be induced at a distance through
76 diffusible or volatile signals, the motility behavior requires close contact and is therefore
77 controlled in a manner distinct from the other two forms of communication.

78 In this study, we describe an interaction between two distantly-related soil bacteria, *P.*
79 *fluorescens* Pf0-1 (phylum: Proteobacteria) and *Pedobacter* sp. V48 (phylum: Bacteroidetes).
80 This interaction produces an emergent behavior, which we term "interspecies social spreading,"

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81 in which the bacteria move together across a hard agar surface. When grown in isolation,
82 neither species moves beyond the typical amount of colony expansion. In co-culture, both
83 bacteria are present throughout the spreading colony, and fluorescent imaging shows a non-
84 homogenous distribution. We demonstrate that a close association between the colonies of both
85 species is required for spreading to initiate and that the levels of nutrients and salts in the media
86 affect the development of the spreading phenotype.

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87 # Results

88 **Interspecies social spreading arises when mixing two distantly-related bacteria**

89 In previous studies, antifungal activity was observed when *P. fluorescens* Pf0-1 and
90 *Pedobacter* sp. V48 were cultured 15 mm apart (6). In addition to this interaction-induced trait,
91 the possibility of motility was noted, but not further investigated, in a mixture of *P. fluorescens*
92 AD21 and *Pedobacter* (5, 9). To explore this phenomenon, we developed an assay in which the
93 induced motility is greater and more easily observed. Our approach differed from the conditions
94 of the original observation in inoculation method, strain combination, and media composition.
95 When we plated *P. fluorescens* Pf0-1 and *Pedobacter* on TSB-NK medium solidified with 2%
96 agar a mixed colony of the two bacteria expanded across the surface of the agar, an
97 environment in which neither monoculture exhibited motility. The emergent social spreading is
98 shown in Fig. 1.

99 Social spreading becomes apparent between 24 and 48 h after inoculation, when the
100 colony begins to spread from the edge of the inoculum (Fig. 1b 48 h). The diameter of the
101 spreading co-culture is significantly different from the colony expansion of the mono-cultures
102 starting at the 24 h time point ($p < 0.001$) (Fig. 1a). Once the spreading phenotype is fully visible
103 (around 72 h), the average speed of expansion is 1.69 μm/min +/- 0.09 (SEM). At the onset of
104 movement, the leading edge has a visibly thicker front (Fig. 1b 48 h). As the colony spreads, the
105 thick front disappears and small 'veins' radiating from the center develop. Over time, the 'veins'
106 become more pronounced towards the leading edge, making a 'petal' pattern (Figs. 2a, b). The
107 leading edge is characterized by a distinctive, terraced appearance comprised of three to six
108 layers (Fig. 2c). Varying the initial ratios between 5:1 and 1:5 *Pseudomonas*:*Pedobacter* did not
109 have a visible effect on spreading across the plate (data not shown).

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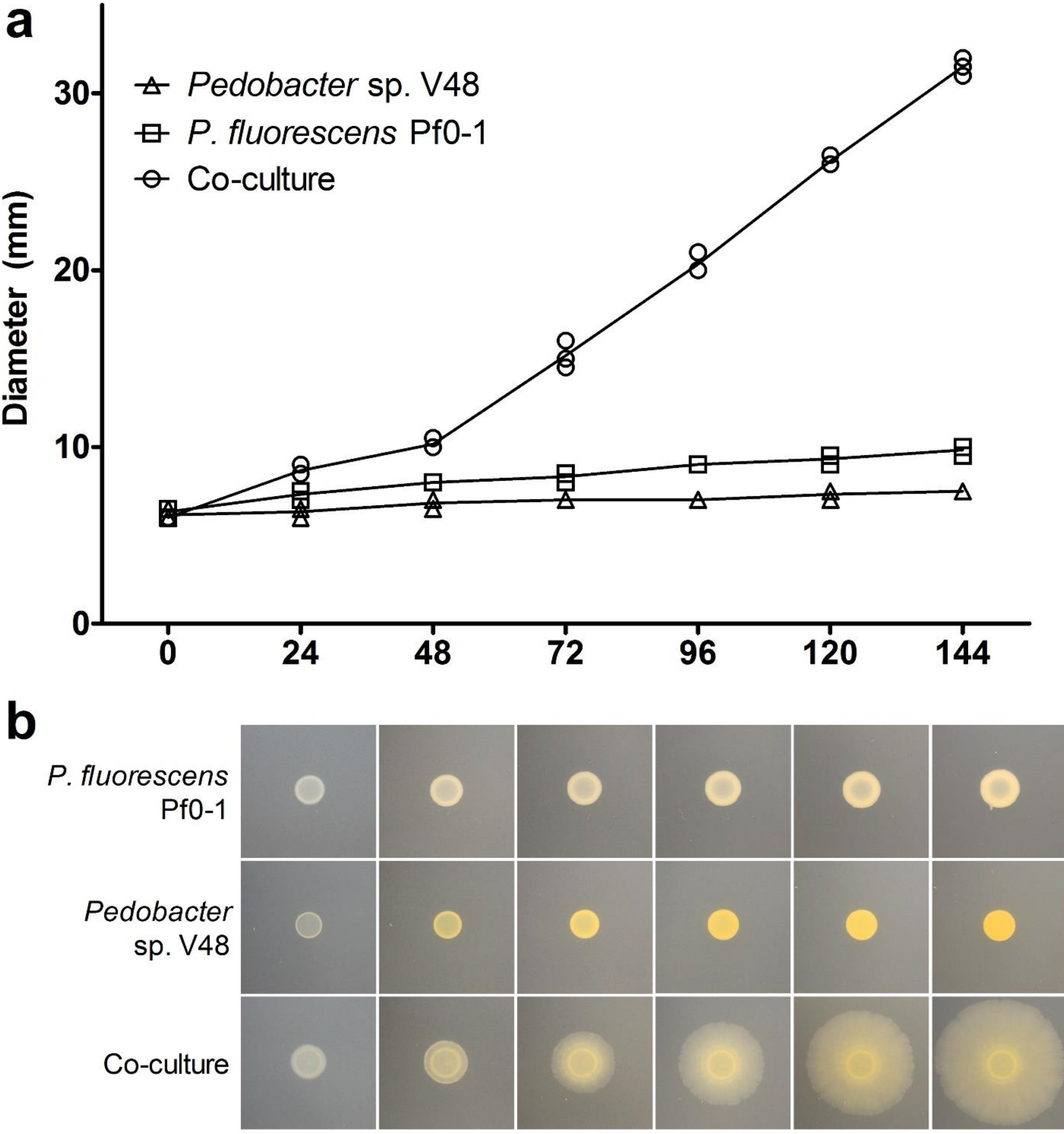

Figure 1. Mixed colony of *P. fluorescens* Pf0-1 and *Pedobacter* sp. V48 spreads across a hard surface (2% agar), a behavior not observed in the mono-culture of either species. a) Diameter of colonies at 24 h intervals for three independent experiments. b) Phenotypes of mono- and co-cultures at 24 h intervals. Contrast and brightness levels were adjusted for optimal viewing.

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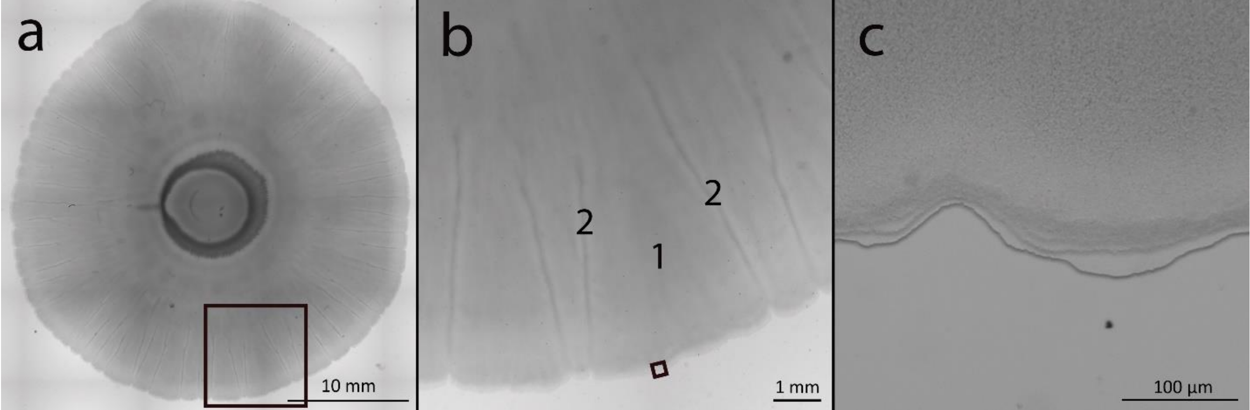

Figure 2. Mixed colony of *P. fluorescens* Pf0-1 and *Pedobacter* sp. V48 at different magnifications. a) Image of the whole co-culture colony created by stitching an 8X magnification mosaic. b) 16X magnification of the leading edge showing the patterns of 'petals' (1) in between 'veins' (2) visible near the edge of the colony c) 112X magnification shows a terraced appearance of the leading edge. Colony imaged 144 h after inoculation. Black boxes indicate area enlarged in the adjacent panel. Scale bars are noted at the bottom of each image. Contrast and brightness levels were adjusted for optimal viewing.

110 ***P. fluorescens* Pf0-1 and *Pedobacter* sp. V48 co-migrate**

111 The previously observed 'bacterial expansion' of *Pedobacter* when interacting with
112 *Pseudomonas* sp. AD21 was suggested to be gliding motility, triggered as a mechanism to
113 escape competition from *P. fluorescens* (5, 9). We examined the possibility that the spreading
114 observed when co-inoculating *Pedobacter* and *P. fluorescens* Pf0-1 was a result of *Pedobacter*
115 moving away from *P. fluorescens*. Bacteria were collected from the center, middle, and edge of
116 a seven-day-old motile colony. The presence or absence of each species was tested by
117 culturing these samples on selective media. We recovered both species from each point in the
118 spreading colony (data not shown), showing co-migration rather than an escape strategy by
119 *Pedobacter*.

120 **Interspecies social spreading shows reproducible spatial organization**

121 To obtain a more detailed look at the spatial relationships within the spreading colony,
122 we tagged *P. fluorescens* with a cyan fluorescent protein (eCFP (21)) and *Pedobacter* with a red
123 fluorescent protein (dsRedEXPRESS (21)), integrated into the chromosome. In *P. fluorescens*,

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124 eCFP carried by a miniTn*7* transposon was integrated upstream of *glmS* (22), creating Pf0-*ecfp*.
125 In *Pedobacter*, dsRedEXPRESS carried by the *HimarEm* transposon (23) was integrated at
126 random locations in the chromosome, resulting in 16 independently-derived mutants with an
127 insert. Each tagged *Pedobacter* strain (V48-*dsRed)* was indistinguishable from the wild-type in
128 social assays with *P. fluorescens*, indicating no deleterious impact of the insertions. We picked
129 one strain with an insert in locus N824_RS25465 (GenBank accession NZ_AWRU01000034),
130 and no apparent defect in social spreading. The initiation of social spreading appeared slightly
131 delayed in a mixture of the tagged strains, but the visible patterns and stages of development
132 looked identical, and speed was not significantly different once spreading initiated.

133 Fluorescent microscopy verified culturing data that showed both bacteria are present
134 throughout the spreading colony, but we also found that population density varies across distinct
135 areas within the colony. These distribution patterns were highly reproducible and show six
136 distinct zones (Fig. 3). At zone 1, the point of inoculation, fluorescent imaging shows a
137 homogenous mix of both bacteria (Fig. 3b, c). Zone 2, the coffee ring effect formed at the edge
138 of the point of inoculation (24–26), is bright orange, indicating that *Pedobacter* dominates this
139 region (Fig. 3b). *Pedobacter* spreads out from this dense area into zone 3, in a starburst pattern
140 (Fig. 3b). Just outward from the starburst, we see a blue ring (zone 4), where *P. fluorescens*
141 appears more abundant (Figs. 3c, d). In the main body of the co-culture, a thin motile section
142 spreads out, making ‘petals’ (Zone 5), with ‘veins’ (Zone 6) between them (Figs. 2b and 3a).
143 The ‘veins’ between the ‘petals’ appear to have high *Pedobacter* populations (Fig. 3b), while the
144 areas directly surrounding them are dominated by *P. fluorescens* (Fig. 3c). The flat areas of the
145 ‘petals’ appear more well-mixed, though the red signal becomes difficult to detect toward the
146 edge of the colony (Fig. 3d). Overall, imaging data show that we can find both species
147 throughout the colony, but the distribution is not homogenous. Rather, we observed
148 reproducible patterns with some well-mixed areas and others of high spatial assortment.

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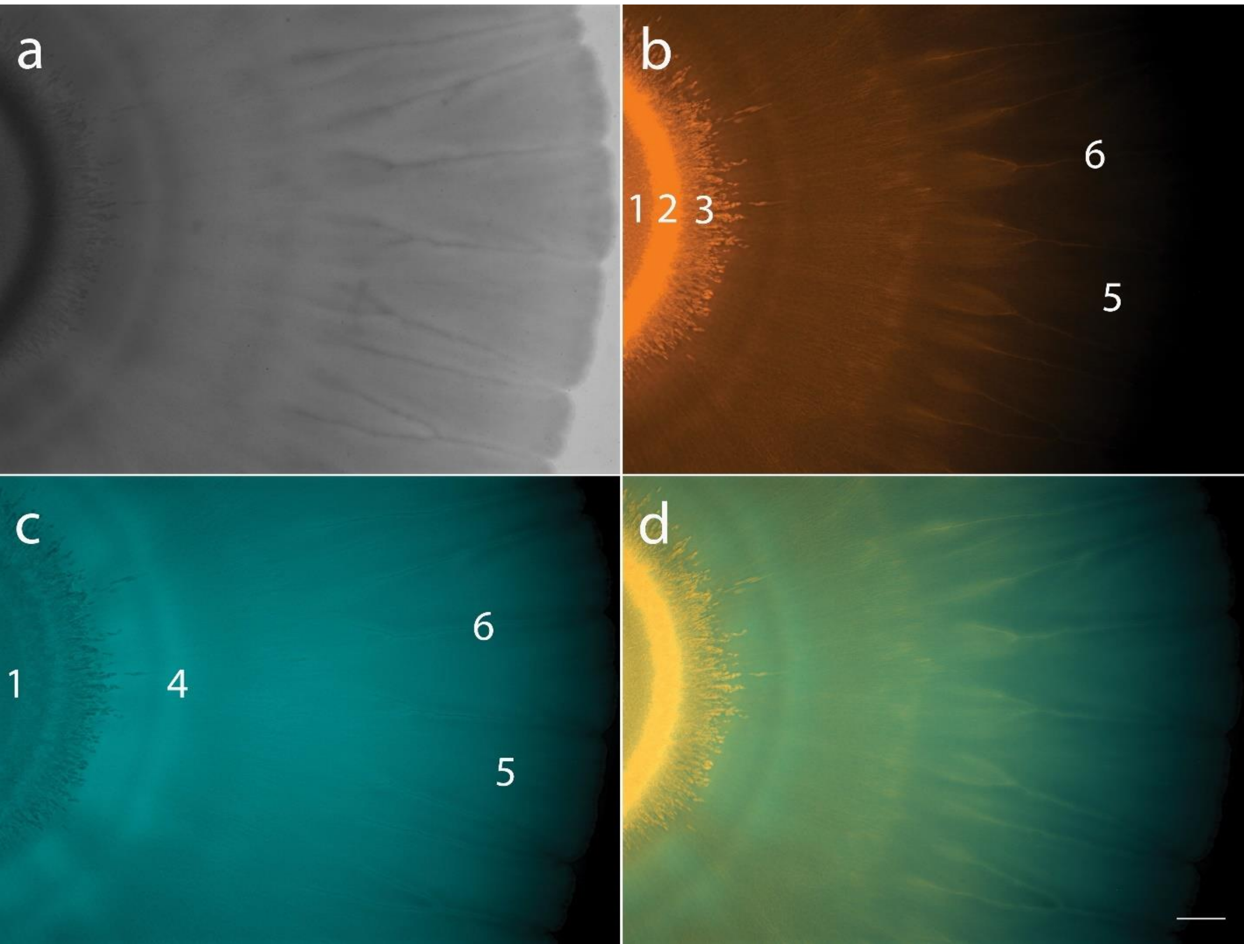

Figure 3. Mixed colony of fluorescently-tagged *P. fluorescens* Pf0-1 (Pf0-*ecfp*) and *Pedobacter* sp. V48 (V48-*dsRed*). a) Co-culture colony viewed with white light. b) Co-culture imaged using DsRed filter (filter set 43 HE), pseudo-colored in orange, showing V48-*dsRed* distribution throughout the colony. c) Co-culture imaged using CFP filter (filter set 47 HE), pseudo-colored in turquoise, showing Pf0-*ecfp* distribution throughout the colony. d) Merged images of DsRed and CFP filters. Numbers on panels b and c indicate six zones of distinct patterns: 1. Point of inoculation, 2. Coffee ring, 3. Starburst, 4. *P. fluorescens* ring, 5. Petals, 6. Veins. Colonies imaged at 7X magnification, scale bar represents 1 mm. Colony imaged 144 h after inoculation.

149 **Diffused compounds and heat-killed cells do not trigger interspecies social spreading**

150 Previous studies demonstrated that interactions between *P. fluorescens* and *Pedobacter*
151 were mediated via both diffusible and volatile signals (6–8). We first asked whether spreading
152 could be triggered by diffusible compounds produced by one of the partner species or by the co-
153 culture. Mono and co-cultures grown on cellophane membranes were used to pre-condition our
154 spreading assay agar. After two days, cellophane membranes (and the bacteria growing on

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155 them) were peeled off the agar. Plates were then inoculated with one of the partner strains to
156 evaluate development of the social spreading phenotype. After seven days of growth, no sign of
157 spreading beyond normal colony expansion was observed (change in diameter was not
158 significantly different from negative control), indicating that no motility-inducing compounds had
159 been secreted into the agar.

160 As the signal did not appear to diffuse through cellulose, we next asked if inactive cells
161 or cell fragments of each species could trigger spreading in the other species. To address this,
162 we used dead cells from one species, or from the spreading co-culture. Mono- and co-cultures
163 were grown on TSB-NK media (as previously described) for 4 days, suspended in phosphate
164 buffer, and heat-killed at 65 °C for 15 minutes. This heat-killed suspension was added directly
165 on top of growing colonies of each species, or to wells adjacent to the colony being tested.
166 Heat-killed suspension was added every 24 hours for five days. The plates were monitored for
167 ten days, but no social spreading was observed under any condition, beyond that due to
168 physical disruption which is also present in the buffer control.

169 **Physical association of *P. fluorescens* Pf0-1 and *Pedobacter* V48 is required for**
170 **interspecies social spreading**

171 Because a diffusible signal was unlikely to be triggering social spreading, we asked
172 whether a close association between the two bacteria was a necessary condition for the
173 interaction. To answer this question, we used assays in which the bacterial participants were
174 plated side-by side with no physical barrier and in which they were separated only by semi-
175 permeable membranes.

176 When colonies were adjacent, rather than mixed, no social spreading was observed
177 while the *P. fluorescens* and *Pedobacter* colonies were visibly separate (data not shown).
178 However, once the colonies grew sufficiently to make contact (Fig. 4 24 h), the colony started to

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179 spread out from the point of contact (72 h). The spreading front radiates outward (96 h), first
180 developing around the *P. fluorescens* colony (144 h), then proceeding to surround the
181 *Pedobacter* colony (192 h). At this level of resolution, contact between the colonies appears to
182 occur before any social spreading can be seen.

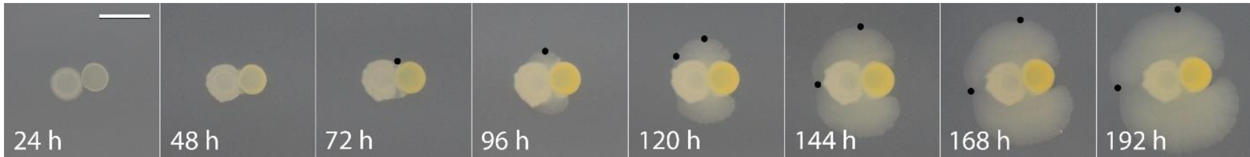

Figure 4. Social spreading emerges after contact between colonies of *P. fluorescen*s Pf0-1 and *Pedobacter* sp. V48, left and right in each panel, respectively. Colonies come into contact 24 hours after inoculation; the motile front becomes visible 48 hours after contact and spreads outward and around the *P. fluorescens* colony before surrounding the *Pedobacter* colony. Black spots indicate sampling locations. Pictures taken every 24 h. Scale bar represents 10 mm.

183 Samples were collected from the edge of the moving front every 24 hours after contact,
184 both on a y-axis from the point of contact and following the moving front as it wrapped around
185 the *P. fluorescens* colony (Fig. 4). The presence of each species was tested by culturing these
186 samples on selective media. Both species were culturable at every point sampled (data not
187 shown), showing that *Pedobacter* is present in the moving front behind the *P. fluorescens*
188 colony (Fig. 4, 144 h), on the opposite side of where they initially came into contact. This
189 indicates that *Pedobacter* moves around the *P. fluorescens* colony on the motile front.

190 To further evaluate the requirement that *P. fluorescens* and *Pedobacter* be physically
191 associated, we inoculated both strains immediately adjacent to each other but separated by
192 either semi-permeable mixed-ester cellulose or PES (polyethersulfone) membranes. When
193 inoculated this way, individual colony growth continued as normal, but these bacteria were
194 unable to trigger social spreading despite their close proximity. After six days of growth, no sign
195 of interspecies social spreading was observed (Fig. 5).

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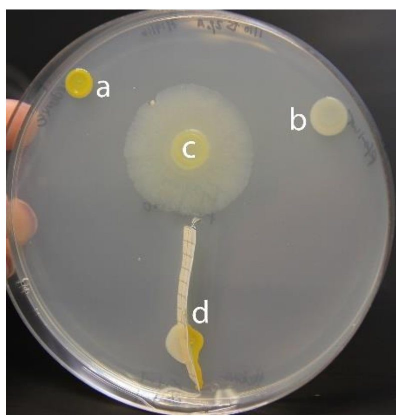

Figure 5. A semi-permeable barrier prevents development of the interspecies social spreading phenotype. a) *Pedobacter* sp. V48 monoculture, b) *P. fluorescens* Pf0-1 monoculture, c) a mixed colony, and d) *P. fluorescens* and *Pedobacter* separated by a mixed-ester cellulose membrane. Pictures taken 144 h after inoculation. Colonies were grown on a 100 mm petri dish.

198 **Nutritional environment influences interspecies social spreading**

199 Conditions in soil and rhizosphere environments fluctuate, with bacteria subjected to a
200 wide range of environmental stressors, including limited nutrient and water availability (2).
201 Because such fluctuations may influence expression of traits, we examined the effect of nutrient
202 level on interspecies social spreading. Our standard assay condition, TSB-NK, consists of 10%
203 strength Tryptic Soy (3 g/L) supplemented with NaCl (5 g/L) and KH_2PO_4 (1 g/L).

204 We first asked if interspecies social spreading could initiate under richer nutrient
205 conditions. No social spreading was apparent when *P. fluorescens* and *Pedobacter* were mixed
206 on full-strength TSB (30 g/L) (Fig. 6a), with the co-culture exhibiting the same characteristics
207 and colony expansion as the *P. fluorescens* mono-culture. We next asked whether the salt
208 amendments to TSB-NK influence interspecies social spreading, using assays without the
209 addition of salts, and with the addition of NaCl and KH_2PO_4 individually. When grown on 10%
210 TSB, the co-culture is motile, but the distance spread is modest compared to when the medium
211 is supplemented with both salts (Fig. 6c). The individual *P. fluorescens* colony expands similarly
212 to the co-culture, suggesting minimal social behavior under these conditions. Growth on TSB-K
213 changes neither pattern nor rate of mono- and co-culture expansion compared to 10% TSB
214 (data not shown). On TSB-N, the mixed culture spreads and develops the patterns
215 characteristic of interspecies social spreading, while the *P. fluorescens* mono-culture does not

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216 expand (Fig. 6d). The phenotype and diameter of the spreading colony are most similar to those
217 observed in TSB-NK conditions (Fig. 6b).

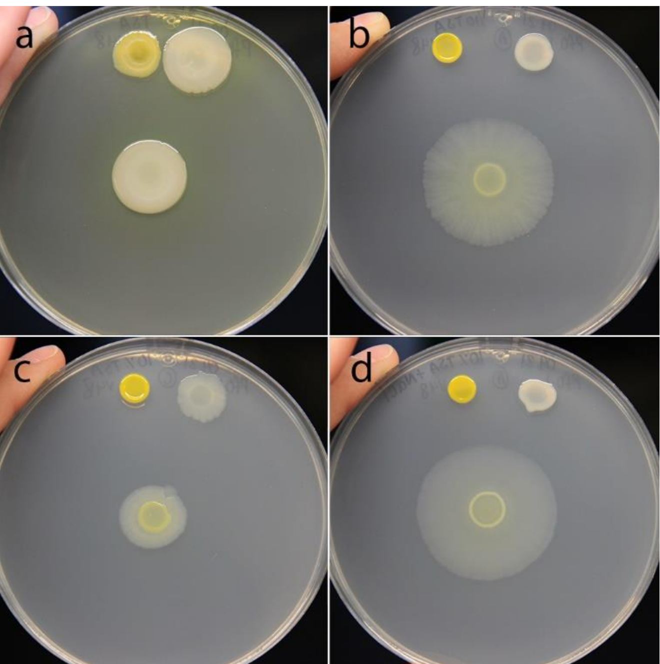

Figure 6. Low nutrient and high salt conditions are required for interspecies social spreading. a) Mixed colony on full-strength TSB does not show social spreading. b) Mixed colony on TSB-NK (10% Tryptic Soy supplemented with both NaCl and KH_2PO_4) shows social spreading. c) Mixed colony on 10% strength TSB shows impaired social spreading. d) Mixed colony on TSB-N (supplemented with NaCl) exhibits the interspecies social spreading phenotype. For all panels *Pedobacter* sp. V48 mono-culture is on the top left of the plate, *P. fluorescens* Pf0-1 is on the top right of the plate, and the mixed colony is in the center. Pictures were taken 144 h after inoculation. Colonies were grown on a 100 mm petri dish.

219 In the previous experiment, we observed that variations of Tryptic Soy media led to
220 altered social phenotypes. To assess the influence of each component of TSB on interspecies
221 social spreading, we utilized a medium in which these were individually manipulated. We made
222 eight combinations of media to vary D-glucose, tryptone, and NaCl in concentrations equivalent
223 to those in full-strength and 10% TSB. On media with D-glucose or tryptone at full-strength
224 concentrations, we did not observe social spreading regardless of the concentration of the other
225 components (Figs. 7a-f). In these conditions, the appearance and expansion of the co-culture
226 resembled that of the *P. fluorescens* mono-culture, with notably greater biomass in media with
227 full-strength tryptone (Figs. 7a-d). When the concentration of all three components was reduced
228 to 10% we observed social spreading, but the migration distance of the co-culture was modest,
229 and *P. fluorescens* mono-culture expanded to a similar extent (Fig. 7h). On media containing
230 10% strength D-glucose, 10% strength tryptone, and full-strength NaCl, interspecies social

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231 spreading emerged when *P. fluorescens* and *Pedobacter* were co-cultured (Fig. 7g). Unique to
232 this condition, the mono-cultures of both strains are immotile, indicating a dramatic change in
233 behavior when strains are mixed. The observations under this condition are most similar to
234 those observed on TSB-N and TSB-NK (Figs. 6b, d).

235 Based on these results, we conclude that full interspecies social spreading was only
236 observed in low nutrient medium supplemented with NaCl (Figs. 6b, d, and 7g). We observed
237 reduced social spreading on low nutrient media without salt supplementation (Figs. 6c and 7h),
238 and an absence of social behavior on rich media (Figs. 6a and 7a-f). While we can implicate salt
239 as an important factor in social spreading, high salt concentrations alone are not sufficient to
240 induce social behavior, as we do not see social behavior under rich media conditions. This
241 indicates that there may be more than one important nutritional component factored in the
242 decision of these bacteria to socialize.

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	Glucose	Tryptone	NaCl	Phenotype
a	High	High	High	
b	High	High	Low	
c	Low	High	High	
d	Low	High	Low	
e	High	Low	High	
f	High	Low	Low	
g	Low	Low	High	
h	Low	Low	Low	

Figure 7. Effect of nutrient levels in interaction between *P. fluorescen*s Pf0-1 and *Pedobacter* sp. V48, looking at 3 core components of TSB: tryptone (20 g/L), D-glucose (2.5 g/L), and NaCl (5 g/L) for 'high' concentrations. Components were reduced to 1/10 for 'low' concentrations. For all panels *Pedobacter* mono-culture is on the top left of the plate, *P. fluorescens* is on the top right of the plate, and the mixed colony is in the center. Pictures were taken 144 h after inoculation. Colonies were grown on 100 mm petri dishes.

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255 **Discussion**

256 In this study we investigate interspecies social spreading, a phenomenon that emerges
257 from the interaction of two distantly-related soil bacteria. Neither species moves on its own
258 under the conditions of our study, but a mixture of the two species can spread across a hard
259 surface (2% agar). Contact between the two bacterial colonies is required for spreading to
260 initiate, and this association is maintained as the co-culture expands. The social phenotype
261 could be observed only under specific nutritional conditions, indicating an interplay between
262 environmental and biological factors. The interaction between *Pedobacter* and *P. fluorescens*
263 serves as a simple and tractable model for investigating interspecies interactions. Our research
264 contributes to the growing body of work studying bacteria in social contexts to investigate
265 emergent traits and behaviors.

266 Surface motility is a trait that could be beneficial to bacteria under a range of
267 environmental conditions. Species related to *Pedobacter* sp. V48 use gliding motility on 1% agar
268 or glass surfaces (27–29). V48 has not been observed to engage in gliding motility, but we have
269 observed phenotypes similar to sliding motility in other species (30, 31), when inoculated on
270 semi-solid agar (unpublished observations). *P. fluorescens* Pf0-1 is capable of flagella-driven
271 swimming in and swarming motility on semi-solid agar (0.3% and 0.6% respectively) without the
272 need for a partner bacterium (32, 33). Interspecies social spreading is distinct from
273 *Pseudomonas* flagellar motility in its requirement of the presence of a second species.
274 Additionally, media with higher agar percentages form environments that are non-permissive for
275 flagella-driven motility in *P. fluorescens*, as well as most species, but together, Pf0-1 and V48
276 appear to employ an alternative strategy for movement across hard surfaces.

277 De Boer *et al.* (5) suggested that in water agar, the sporadic occurrence of movement
278 they observed indicated a strategy by *Pedobacter* to escape competition. However, the co-

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279 migration under our conditions does not support this hypothesis, as the two species remain
280 associated throughout the colony. Our contact experiments provide further evidence, as the
281 presence of *Pedobacter* in the motile areas surrounding the *P. fluorescens* colony shows it has
282 moved towards its partner, rather than away from it. The pattern of *Pedobacter* migration clearly
283 indicates that it is not escaping.

284 Evidence, both from culturing and fluorescent imaging, shows that *P. fluorescens* and
285 *Pedobacter* co-migrate across the hard agar surface. Initiation of the process requires physical
286 contact, as motility is precluded when a semi-permeable membrane is placed between the two
287 colonies. We suggest that the nature of this interaction is distinct from contact-dependent toxin
288 delivery systems, such as type VI secretion and contact-dependent growth inhibition, as they
289 commonly mediate signal exchange between closely-related species, and are involved in
290 competition between more distantly-related strains (34–36). While our results do not rule out
291 quorum sensing for communication between the two species (37), a diffusible signal (if it exists)
292 does not appear be sufficient to trigger the motility response. Additionally, our experiment in
293 which bacteria are pre-grown on cellophane indicates that social spreading is not triggered by a
294 change in the medium caused by metabolic activity of one of the two species. Our data indicate
295 that physical association is required for social spreading between *P. fluorescens* and
296 *Pedobacter*. The question remains, are the bacteria producing a signal which induces an
297 already-present motility mechanism in one species, or are they directly manipulating the
298 environment in a way which facilitates co-migration, such as by production of a surfactant?
299 Regardless of which mechanism is used, close association is still a prerequisite for either
300 induction or facilitation of social spreading.

301 Bacteria dwelling in soil experience variations in a wide range of abiotic conditions,
302 including the key parameters we have tested: salinity and available carbon and nitrogen (2).
303 Environmental conditions have previously been shown to affect motility of individual species;

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304 gliding motility in some *Flavobacterium* species increases with reduced nutrient concentration
305 (38, 39). Changes in behavior resulting from environmental fluctuations can affect how species
306 interact with one another. The ability of *P. fluorescens* and *Pedobacter* to spread socially is
307 dependent upon the conditions in which they are growing. In general, high concentrations of
308 glucose and amino acids lead to a build-up of biomass and no apparent social movement.
309 Lower glucose and amino acid concentrations are associated with interspecies social spreading
310 across the plate, but decreasing the salt concentration of the media slows expansion of the
311 colony. Social spreading resulting from the interaction is conditional, with alteration of just a
312 subset of environmental factors resulting in dramatic changes in behavior. It is tempting to
313 speculate that the consortium of *P. fluorescens* and *Pedobacter* can integrate signals from each
314 other's presence and from the nutrient conditions of their environment to determine whether to
315 behave socially. We see similar examples of intraspecies social behaviors being influenced both
316 by biotic factors (quorum sensing) and by abiotic factors (nutrient conditions) in *P. aeruginosa*
317 (40), *Bacillus subtilis* (41), and yeast (42).

318 There are a wide variety of examples of motility resulting from interspecies interactions,
319 where the presence of a motile partner fosters the motility of an immotile participant. Non-motile
320 *Staphylococcus aureus* hitchhikes on swimming *P. aeruginosa* (43) and *Burkholderia cepacia*
321 co-swarms with *P. aeruginosa* in environments where it cannot do so independently (19). *X.*
322 *perforans* induces motile *P. vortex* to swarm towards it, which allows it to hitchhike on top of *P.*
323 *vortex* rafts (20). *P. vortex* is also capable of carrying fungal spores or antibiotic-degrading
324 cargo bacteria to cross unfavorable environments (18, 44). In an even more complex system,
325 *Dyella japonica* can migrate on fungal hyphae, but some strains can only do so in the presence
326 of a *Burkholderia terrae* helper (45, 46). All of these examples of 'hitchhiking' phenomena
327 require one species to already be motile, and stand in contrast to the behavior we have
328 investigated, where social spreading emerges from two conditionally non-motile participants.

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329 The fact that both species are present at the edge of the spreading colony suggests that both
330 have an active role in the behavior, though it doesn't rule out the possibility of one species
331 inducing motility in the other and hitchhiking, as seen in other systems (20).

332 In addition to describing a new mode of motility, this discovery highlights the possibility
333 that many functions and behaviors of bacteria in complex communities may be triggered by
334 interactions between different species or even domains. Studying interactions between two or
335 more microorganisms may lead to the discovery of emergent traits that would be impossible to
336 predict based on the study of each organism in isolation. Alongside approaches that
337 characterize the members and connectedness of microbial communities, tools to decipher the
338 phenotypic outcomes of interactions are needed in order to develop a full appreciation of
339 microbiomes. Studies of this type are important for understanding the role of microbial
340 communities within an ecological context.

341 We have investigated an interaction-dependent trait which emerges under particular
342 nutritional conditions when distantly-related bacteria come into close physical contact. This
343 interaction gives the participating bacteria the ability to spread on a hard agar surface, which
344 neither can do alone. This strategy of co-migration may serve as an additional mechanism by
345 which plant- and soil-associated bacteria can move in their natural environments, when the
346 conditions do not favor the modes of single-species motility previously described. Given the
347 distant and different locations from which these two strains were isolated, we hypothesize this is
348 not a unique interaction between this pair, but rather has evolved between various *Pedobacter*
349 and *Pseudomonas* species. To understand the phenomenon, several lines of investigation
350 should be pursued: mechanistic studies which explore the factors each species is contributing to
351 social spreading, the process by which contact triggers motility, whether there are important
352 metabolic interactions, and the way in which environmental conditions are integrated into the
353 decision to move together. The system we study is a tractable model for studying interspecies

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354 interactions, giving us the opportunity to answer questions about the nature of interspecies
355 social spreading and ask questions about the broader field of bacterial communities. Models
356 such as these will ultimately lead to a greater understanding of the functions of communities as
357 a whole rather than as collections of individuals.

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358 ## Materials and Methods

359 **Bacterial strains, primers, plasmids, and culture conditions.** Bacterial strains and plasmids
360 are described in Table 1. *E. coli* was grown at 37 °C in LB Broth, Miller (Fisher Scientific).
361 *Pseudomonas fluorescens* Pf0-1 and *Pedobacter* sp. V48 were routinely grown at 30°C or 20°C
362 respectively, in 10% strength Tryptic Soy Broth (BD Difco™) amended with NaCl and KH_2PO_4,
363 as described by de Boer (5). This medium is referred to throughout the text as TSB-NK. To
364 differentiate the two species from mixed cultures we used *Pseudomonas* minimal medium
365 (PMM) with 25 mM succinate (47) for *P. fluorescens* and 14.6 mM lactose for *Pedobacter*.
366 Media were solidified with BD Difco™ Bacto™ agar (1.5% w/v) when required, except for social
367 spreading assays, for which 2% agar was used. For experiments with variations in nutrients, we
368 used full-strength TSB (30 g/L), 10% TSB (3 g/L), and 10% TSB amended with NaCl or KH_2PO_4
369 (called TSB-N or TSB-K, respectively), and a medium composed of D-glucose (2.5 g/L),
370 tryptone (20 g/L), and NaCl (5 g/L). These individual components were used at those
371 concentrations or reduced to 10% concentration in all eight combinations. For selection of
372 transposon insertions carrying fluorescent protein genes, Kanamycin (50 µg/mL), Gentamicin
373 (50 µg/mL), or Erythromycin (100 µg/mL) was added to the growth medium.

374 **Interspecies social spreading assays.** *P. fluorescens* and *Pedobacter* for use in social
375 spreading assays were incubated in 2 mL TSB-NK at 20 °C for 24 hours, with shaking (160
376 rpm). Social spreading assays were carried out on TSB-NK solidified with 2% agar. Plates were
377 poured at a temperature of ~62 °C in a single layer and allowed to set for ~15 minutes before
378 inoculation. Inoculation was done on freshly-poured plates.

379 (i) Mixed inoculum assays. Assays were started by combining 5 µL of each participant in one
380 spot on the agar surface. As controls, 10 µL spots of each bacterial isolate were plated distant
381 from each other and the co-culture, all on the same plate. Once the inoculation liquid had dried,

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382 plates were incubated at 20 °C. Measurements of the colony diameter were taken every 24
383 hours. Experiments were performed in triplicate.

384 (ii) Direct contact assay - adjacent plating. *P. fluorescens* and *Pedobacter* were grown as
385 described above. The aliquots of bacteria were plated adjacent but without the drops touching.
386 Once the inoculation liquid had dried, plates were incubated at 20 °C and monitored daily to
387 determine the time at which colony growth led to contact between the isolates, and when
388 spreading phenotypes developed.

389 (iii) Direct contact assay - separation by membranes. *P. fluorescens* and *Pedobacter* were
390 plated close together, separated only by a membrane. Either Millipore Polyethersulfone (PES)
391 Express Plus® Membrane (0.22 µm pores) or Gelman Sciences mixed-ester cellulose Metricel
392 Membrane (0.45 µm pores) were cut into rectangular strips and sterilized by autoclaving. These
393 strips were then embedded into the agar by suspending them perpendicular to the bottom of
394 petri dishes with forceps, as agar was poured into plates. Once set, the filters protruded
395 approximately 5 mm above the agar surface. Bacteria were inoculated on either side of the filter,
396 with 5 µL spots of each species, close enough to touch the filter.

397 (iv) Cellophane overlay assay. Squares of porous cellophane (GE Healthcare Bio-sciences
398 Corp) were placed on top of TSB-NK plates. Cultures of *P. fluorescens*, *Pedobacter*, and a co-
399 culture of the two, were placed on top of the cellophane, with cellophane alone used as a
400 negative control. Plates were incubated at 20 °C for two days, at which point cellophane was
401 removed, and 5 µL spots of either species were placed in the center of the plate, so that
402 cultures were on a plate where cellophane had been (negative control), one where the partner
403 species had been cultured, or one where a mix of the species had been cultured.

404 (v) Heat-kill assay. Cells were scraped from TSB-NK plates, suspended in PBS buffer, heat-
405 killed, and added on top of or adjacent to a colony of *Pseudomonas* or *Pedobacter* to test the

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406 ability of heat-killed cells to induce movement in the partner species. To place the heat-killed
407 suspension adjacent to living colonies, a well was made in freshly-poured agar, by cutting a
408 core using the top end of a 10 µL pipette tip (USA Scientific, Inc.), and partially filling it in using
409 60-70 µL agar. Cultures of *Pseudomonas* or *Pedobacter* (5 µL spots) were inoculated adjacent
410 to the well, and the well was filled with the heat-killed suspension. For experiments in which the
411 heat-killed suspension was added directly on top of living colonies, these colonies were initiated
412 with 10 uL spots of liquid culture. The suspensions added directly on top of the colony or to the
413 wells were heat-killed *Pseudomonas* or co-culture on/next to a *Pedobacter* colony, or heat-killed
414 *Pedobacter* or co-culture on/next to a *Pseudomonas* colony. These heat-killed cells, or PBS
415 buffer as a negative control, were added to the colonies or wells every 24 hours until the end of
416 the experiment. The cells added on top of the colonies or into the wells were extracted from 4-
417 day-old mono- and co-culture colonies on TSB-NK, inoculated and cultured as previously
418 described. Whole colonies from these plates were resuspended in 1 mL PBS buffer, vortexed
419 until fully suspended, then heat-killed at 65 ^{o}C for 15 minutes. Effectiveness of heat-killing was
420 evaluated by plating 100 µL of resuspension on TSB-NK, and PMM with succinate or lactose.

421 **Fluorescent protein tagging**

422 (i) eCFP labeling of *P. fluorescens*. pUC18T-mini-Tn*7*T-Gm-*ecfp* was a gift from Herbert
423 Schweizer (Addgene plasmid # 65030). A constitutively-expressed fluorescent protein gene
424 carried by pUC18T-mini-Tn*7*T-Gm-*ecfp* was transferred to *P. fluorescens* by conjugation from *E.*
425 *coli* S17-1, with transposase being provided by pUX-BF13 introduced from a second *E. coli*
426 S17-1 donor, as previously described (48). Transposon-carrying strains were selected by
427 growth on Gentamicin (50 µg/mL), and transposition of the miniTn*7* element into the target site
428 in the *P. fluorescens* genome was confirmed by PCR using primers Tn*7*-F and *glmS*-R (Table
429 2). Pf0-1 with fluorescent inserts were tested for alteration in interspecies social spreading by
430 co-culturing with *Pedobacter*, as described above.

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431 (ii) dsRedEXPRESS labeling of *Pedobacter*. pUC18T-mini-Tn*7*T-Gm-*dsRedExpress* was a gift
432 from Herbert Schweizer (Addgene plasmid #65032). To express *dsRedEXPRESS* in
433 *Pedobacter*, a *Pedobacter* promoter was cloned upstream of the *dsRedEXPRESS* coding
434 sequence. A highly expressed gene from an unpublished RNAseq experiment was identified
435 (N824_RS25200) and the upstream 300 bp were amplified from *Pedobacter* genomic DNA
436 using primers P*ompA* and *dsRed*, designed for splicing-by-overlap extension-PCR (SOE-PCR)
437 (Table 2). The promoter was then spliced with the amplified *dsRedEXPRESS* coding sequence
438 using SOE-PCR (49). Flanking primers were designed with *Kpn*I restriction sites, enabling
439 cloning of the spliced product into a *Kpn*I site in *pHimarEm1* (23). To join compatible ends
440 between the plasmid and the amplicons, we used T4 DNA ligase (New England Biolabs, Inc.).
441 The ligated plasmid was introduced into *E. coli* S17-1 competent cells by electroporation
442 (BioRad Micropulser™). S17-1 colonies carrying the plasmid were selected by plating on LB
443 medium containing Kanamycin (50 µg/mL), and the presence of the *dsRedEXPRESS* gene was
444 confirmed by PCR, using *pHimar Kpn*I-flank primers (Table 2). The resulting plasmid is called
445 *pHimarEm1-dsRed*.

446 *pHimarEm1-dsRed* was transferred to *Pedobacter* by conjugation using a method adapted from
447 Hunnicutt and McBride, 2000. Briefly, 20 hour old cultures of *E. coli* S17-1 (*pHimarEm1-dsRed*)
448 and *Pedobacter* were subcultured 1:100 into fresh LB, and grown to mid-exponential phase (*E.*
449 *coli*) or for 7 hours (*Pedobacter*). Cells were collected by centrifugation, suspended in 100 µL of
450 LB, and then mixed in equal amounts on TSB-NK with 100 µL of 1M $CaCl_2$ spread on the
451 surface. Following overnight incubation at 30 °C, cells were scraped off the surface of the plate,
452 and dilutions were plated on TSB-NK with Erythromycin (100 µg/mL) to select for strains that
453 received the plasmid (*ermF* is not expressed in *E. coli*). Transconjugants were incubated at 25
454 °C for 3-4 days. Presence of the transposon insert in *Pedobacter* was confirmed using *ermF*

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455 primers (Table 2). *Pedobacter* with fluorescent inserts were tested for alteration in interspecies
456 social spreading by co-culturing with *Pseudomonas*, as described above.

457 The transposon insertion sites in the *Pedobacter* chromosome were amplified by arbitrarily-
458 primed PCR (51), using a method adapted from O'Toole *et al.* (52) (see table 2 for primers), and
459 identified by sequencing the arb-PCR products. Nucleic acid sequencing was performed by
460 Massachusetts General Hospital CCIB DNA Core. Sequences were analyzed using CLC
461 Genomics Workbench Version 10.1.1 (QIAGEN) to find location of transposon integration.

462 **Imaging.** Still pictures were taken using an EOS Rebel T3i camera (Canon) and processed
463 using Photoshop CC 2017 Version: 14.2.1 and Illustrator CC 2017 Version: 17.1.0 (Adobe).
464 Using Photoshop, the levels of some images were adjusted to improve contrast.

465 For microscopy, motile colonies were examined using an Axio Zoom.V16 microscope (Zeiss).
466 To visualize fluorescent strains, filter set 43 HE DsRed was used with a 1.5 s exposure, shown
467 with pseudo-color orange, as well as filter set 47 HE Cyan Fluorescent Protein, with a 600 ms
468 exposure, shown with pseudo-color turquoise. Images were captured using Axiocam 503 mono
469 camera, with a native resolution of 1936x1460 pixels. For image acquisition and processing we
470 used Zen 2 Pro software (Zeiss).

471 **Statistics.** We measured the amount of colony expansion of the mono-cultures of both *P.*
472 *fluorescens* and *Pedobacter* and the expansion of social spreading in co-culture. Colony
473 diameter of three independent experiments was measured every 24 hours. To compare the
474 diameter of mono-cultures and co-cultures at each time point, we performed a two-way ANOVA
475 followed by a Bonferroni post-hoc test, using GraphPad Prism version 5.04 for Windows
476 (Graphpad Software).

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477 We compared the movement speed between a combination of wild type *P. fluorescens* and
478 *Pedobacter* to a combination of fluorescently-tagged Pf0-ecfp and V48-dsRed. Colony diameter
479 of six independent experiments were measured every day, and speed was calculated by
480 dividing the distance traveled by the amount of time elapsed since the last time point. To
481 calculate average speed, we only used time points after the interspecies social spreading
482 phenotype developed. To compare the means of the speed of the wild-type and tagged strains,
483 we conducted an unpaired, two-tailed, Student's t-test, using GraphPad Prism version 5.04.

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490 **Author contribution statement**

491 LMM designed and carried out experiments, analyzed data, wrote the manuscript. ASB, SCS,
492 and LMS each contributed a key experiment, and edited the manuscript. MWS contributed to
493 experimental design, data analysis, writing and editing of the manuscript.

494 **Competing Interests**

495 The authors declare that they have no competing interests.

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633 **Tables**

634 **Table 1. Bacterial strains and Plasmids**

Strain	***Genotype or Description***	***Source or Reference***
E. coli		
S17-1	*recA thi pro hsdR* RP4-2-Tc::Mu-Km::Tn*7* λ*pir* Sm^r Tp^r	(53)
P. fluorescens		
Pf0-1	Wild type, Ap^r	(54)
Pf0-*ecfp*	Pf0-1::mini-Tn*7 ecfp* Gm^r	This study
Pedobacter		
V48	Wild type	(55)
V48-*dsRed*	V48 N824_RS25465T899::*HimarEm1* P*ompA-dsRedEXPRESS* Em^r NCBI Accession: NZ_AWRU00000000.1, as of September 25, 2017	This study
Plasmids		
pUC18T-mini-Tn7T-Gm-dsRedEXPRESS	Gm^r	(21)
pUC18T-mini-Tn7T-Gm-ecfp	Gm^r	(21)
pUX-BF13	R6K replicon-based helper plasmid carrying Tn*7* transposase genes	(56)
pHimarEm1	Plasmid carrying *HimarEm1*; Km^r (Em^r)	(23)
pHimarEm1-dsRed	pHimarEm1Ω(3.529kb::P*ompA-dsRedEXPRESS)*	This study

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635 **Table 2. Primers**

Primers	***Sequence (5'-3')***	***Purpose***	***Source or Reference***
Tn*7* F	5'-CAGCATAACTGGACTGATTTCAG -3'	Verify integration of transposon into chromosomal *glmS* locus	(48)
glmS R	5'-TGCTCAAGGGCACTGACG-3'	"	(48)
P*ompA-dsRed* F	5'ACGTTCTCGGAGGAGGCCATCAAC GCAACAAAAGAAACTGC 3'	Amplification of N824_RS25200 promoter to join with dsRed gene	This study
P*ompA* R	5'-TATGGTACC AGTCATCTAGGCGGCTGTAG-3'	" Includes KpnI-site for inserting into pHimarEm1	This study
dsRed F	5'-TACTCAGGAGAGCGTTCACC-3'	Amplification of *dsRed* gene with no promoter, to join with V48 N824_RS25200 promoter by SOE PCR	This study
dsRed R	5'-GCAGTTTCTTTTGTTGCGTTGATGGC CTCCTCCGAGAACGT-3'	" Includes KpnI-site for inserting into pHimarEm1	This study
pHimar KpnI-flank F	5'-CTGCCCTGCAATCGACCTCG-3'	Verify ligation of *dsRed* into pHimarEm1	This study
pHimar KpnI-flank R	5'-CAGATAGCCCAGTAGCTGAC-3'	"	This study
erm F	5'-CCGCACCCAAAAAGTTGCAT-3'	Verify integration of transposon into V48 chromosome.	This study
erm R	5'-GACAATGGAACCTCCCAGAA-3'	"	This study
ARB1	5'-GGCCACGCGTCGACTAGTACNNNNN NNNNNGATAT-3'	Find location of transposon integration in V48 chromosome.	(52)
ARB6	5'-GGCCACGCGTCGACTAGTACNNNNN NNNNNACGCC-3'	"	(52)
ARB2	5'-GGCCACGCGTCGACTAGTAC -3'	"	(52)
Himar Arb1 (TnExt)	5'-GTGTTGTTCCAGTTTGAGATC-3'	"	This study
Himar609 Arb2 (TnInt)	5'-TGGGAATCATTTGAAGGTTGG-3'	"	(23)