

## **Social Motility: Interaction between two sessile soil bacteria leads to emergence of surface motility**

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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  
7 the mono-culture of either species, co-culture reveals an emergent phenotype we term ‘social  
8 motility,’ where the bacteria spread across the hard surface. We show that initiation of social  
9 motility requires close association between the two species of bacteria. Both species remain  
10 associated throughout the spreading colony, with reproducible and non-homogenous patterns of  
11 distribution. The nutritional environment influences social motility; no social behavior is observed  
12 under high nutrient conditions, but low nutrient conditions are insufficient to promote social  
13 motility without high salt concentrations. This simple two-species consortium is a tractable  
14 model system that will facilitate mechanistic investigations of interspecies interactions and  
15 provide insight into emergent properties of interacting species. These studies will contribute to  
16 the broader knowledge of how bacterial interactions influence the functions of communities they  
17 inhabit.

## 18 **Introduction**

19           Within the soil live a plethora of microbial species that form complex communities  
20 responsible for important ecological functions, such as nutrient cycling and plant health. Omics  
21 approaches have given us a wealth of information on the composition, diversity, metabolic  
22 potential, and ecology of plant- and soil-associated microbial communities (Reviewed in  
23 Philippot *et al.*, 2013; Fierer, 2017). However, to get a complete understanding of microbial  
24 functions and interactions within these environments, we must look at every layer, from the full  
25 community *in vivo* to the individual microbe *in vitro* (Reviewed in Abreu and Taga, 2016).  
26 Historically, research has focused on the study of single species in pure culture, but bacteria are  
27 social organisms, and thus study of the mechanisms and consequences of multi-species  
28 interactions is necessary for us to understand the function of microbial communities as a whole.  
29 Investigating entire soil communities *in situ* presents considerable challenges because of  
30 fluctuating soil conditions and the wide range of relevant scales, ranging from particulate to  
31 ecological levels (Reviewed in Fierer, 2017). Reducing the microbial community to pair-wise  
32 interactions or small consortia allows for a detailed mechanistic study and is an essential link  
33 between from the study of isolated microbes in the laboratory to understanding the collective  
34 activities of natural microbial communities (Blasche *et al.*, 2017).

35           Recent work has considered the social environment of bacteria, investigating altered  
36 behaviors and production of secondary metabolites when co-cultured with other organisms.  
37 Some bacteria exhibit emergent behaviors when presented with other species, likely the result  
38 of induction of genes that are not expressed in pure culture. For example, *Pseudomonas*  
39 *fluorescens* produce an antifungal compound during interactions with other species (de Boer *et al.*  
40 *et al.*, 2007; Garbeva and de Boer, 2009; Garbeva *et al.*, 2011a, 2011b, 2014). The co-culture of  
41 different actinomycete species results in the production of secondary metabolites, changes in  
42 pigment, and sporulation (Seyedsayamdost *et al.*, 2011; Traxler *et al.*, 2012, 2013). The  
43 presence of *E. coli* or *Pseudomonas* species effects sporulation and biofilm formation in *Bacillus*  
44 *subtilis* (Powers *et al.*, 2015; Grandchamp *et al.*, 2017). One subset of social interactions are  
45 those which alter the motility behaviors and capabilities of other species. For example, physical  
46 association with *Saccharomyces cerevisiae* results in *Streptomyces venezuelae* consuming the  
47 yeast and triggers ‘exploratory growth’ of the bacteria (Jones *et al.*, 2017). In another example,  
48 *B. subtilis* moves away from a *Streptomyces* competitor across a solid surface (Stubbendieck  
49 and Straight, 2015; Liu *et al.*, 2018). Other behaviors appear less competitive, where a motile  
50 species will travel with a non-motile species that can degrade antibiotics, allowing the

51 consortium to colonize hostile environments (Venturi *et al.*, 2010; Finkelshtein *et al.*, 2015).  
52 *Xanthomonas perforans* can even change the behavior of *Paenibacillus vortex*, producing a  
53 signal that induces *P. vortex* to swarm towards it so it can hitchhike (Hagai *et al.*, 2014).

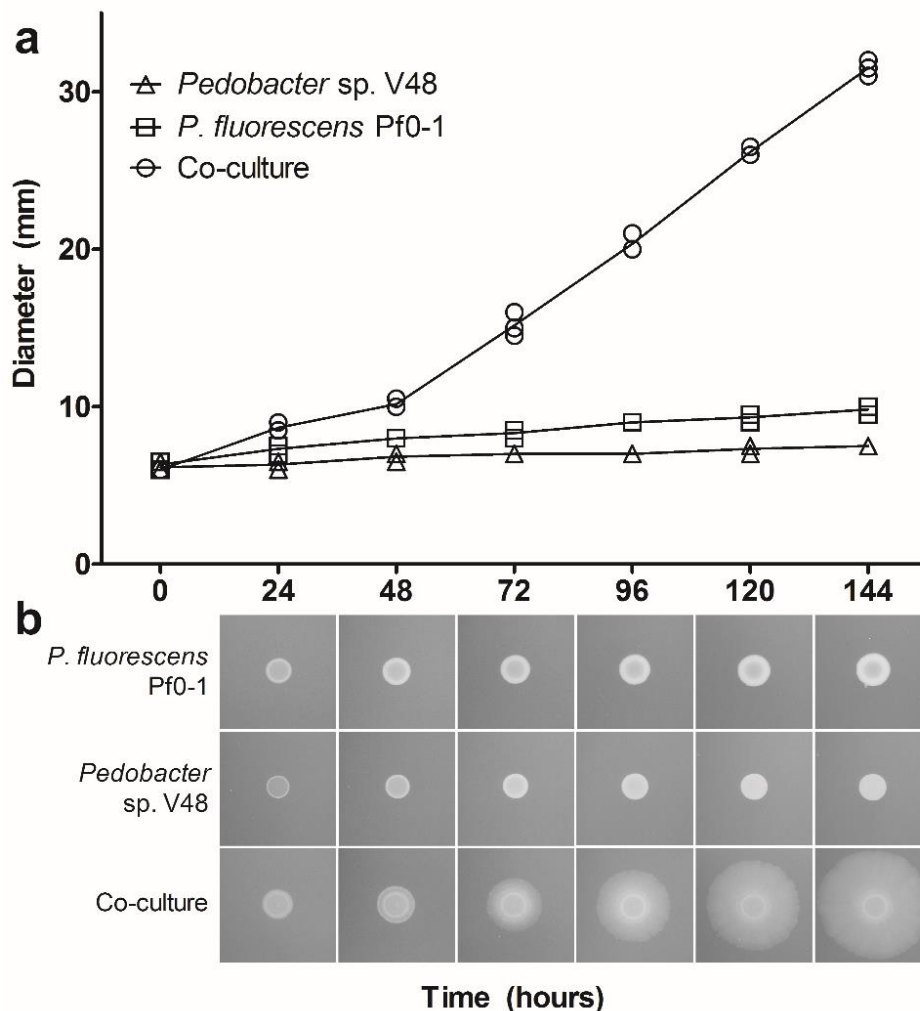
54 *Pseudomonas fluorescens* Pf0-1 and *Pedobacter* sp. V48 are known to interact through  
55 diffusible and volatile signals, which induce changes in gene expression and production of an  
56 antifungal compound by *P. fluorescens* (Garbeva *et al.*, 2011a, 2011b, 2014). Previous studies  
57 with *Pedobacter* and a strain closely-related to *P. fluorescens* Pf0-1 (AD21) found that, in  
58 addition to reciprocal gene expression changes and antagonistic behavior toward the plant  
59 pathogen *Rhizoctonia solani*, the mixture of the strains also showed expansion on the plate  
60 beyond the initial area of inoculation (de Boer *et al.*, 2007; Garbeva and de Boer, 2009). We  
61 further investigated this observed behavior by moving from culturing *P. fluorescens* Pf0-1 and  
62 *Pedobacter* without contact, as was done in the antagonism assays (Garbeva *et al.*, 2011a), to  
63 mixing them together. We hypothesized that, while antibiotic production can be induced at a  
64 distance through diffusible or volatile signals, the motility behavior requires close contact and is  
65 therefore controlled in a manner distinct from the other two forms of communication.

66 In this study, we describe an interaction between two distantly-related soil bacteria, *P.*  
67 *fluorescens* Pf0-1 (phylum: Proteobacteria) and *Pedobacter* sp. V48 (phylum: Bacteroidetes).  
68 This interaction produces an emergent behavior, which we term “social motility,” in which the  
69 bacteria move together across a hard agar surface. When grown in isolation, neither species  
70 moves beyond the normal amount of colony expansion. In co-culture, both bacteria are present  
71 throughout the motile colony, and fluorescent imaging shows a non-homogenous distribution.  
72 We demonstrate that a close association between the colonies of both species is required for  
73 motility to initiate and that the levels of nutrients and salts in the media affect the development of  
74 the motile phenotype.

## 75 **Results**

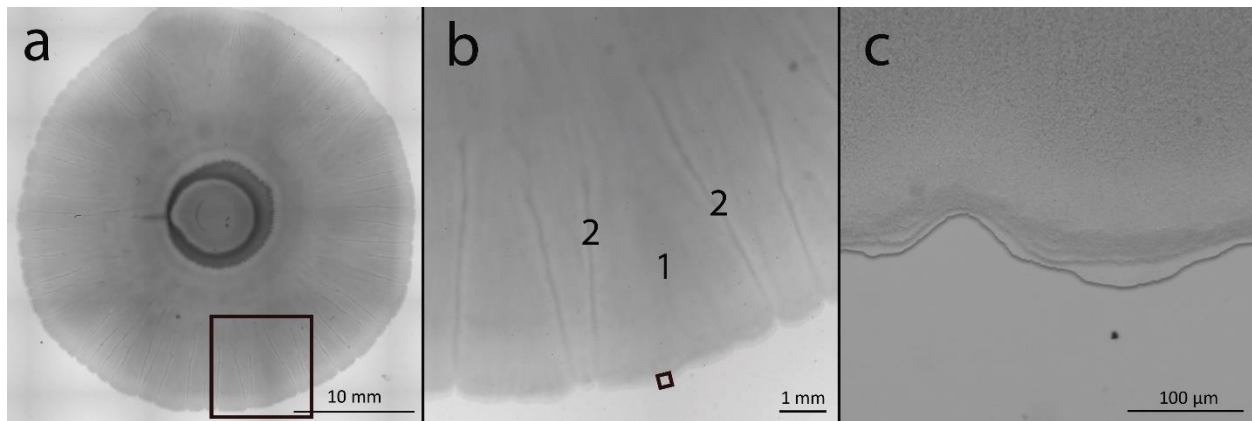
### 76 **Social motility arises when mixing two distantly-related bacteria.**

77 In previous studies, antifungal activity was observed when *P. fluorescens* Pf0-1 and  
78 *Pedobacter* sp. V48 were cultured 15 mm apart (Garbeva *et al.*, 2011a). In addition to this  
79 interaction-induced trait, the possibility of motility was noted in a mixture of *P. fluorescens* AD21  
80 and *Pedobacter* (de Boer *et al.*, 2007; Garbeva and de Boer, 2009). When we plated *P.*  
81 *fluorescens* Pf0-1 and *Pedobacter* on TSB-NK medium solidified with 2% agar a mixed colony  
82 of the two bacteria expanded across the surface of the agar, an environment in which neither  
83 monoculture exhibited motility. The emergent social motility is shown in Fig. 1.



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

84 Social motility becomes apparent between 24 and 48 h after inoculation, when the  
85 colony begins to spread from the edge of the inoculum (Fig. 1b). The diameter of the motile co-  
86 culture is significantly different from the colony expansion of the mono-cultures starting at the 24  
87 h time point ( $p < 0.001$ ) (Fig. 1a). Once the motility phenotype is fully visible (around 72 h), the  
88 average speed of expansion is  $1.69 \mu\text{m}/\text{min} \pm 0.09$  (s.e.m). At the onset of movement, the  
89 leading edge has a visibly thicker front (Fig. 1b 48 h). As the colony spreads, the thick front  
90 disappears and small 'veins' radiating from the center develop. Over time, the 'veins' become  
91 more pronounced towards the leading edge, making a 'petal' pattern (Figs. 2a, b). The leading  
92 edge is characterized by a distinctive, terraced appearance comprised of three to six layers (Fig.  
93 2c).



**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. White 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.

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

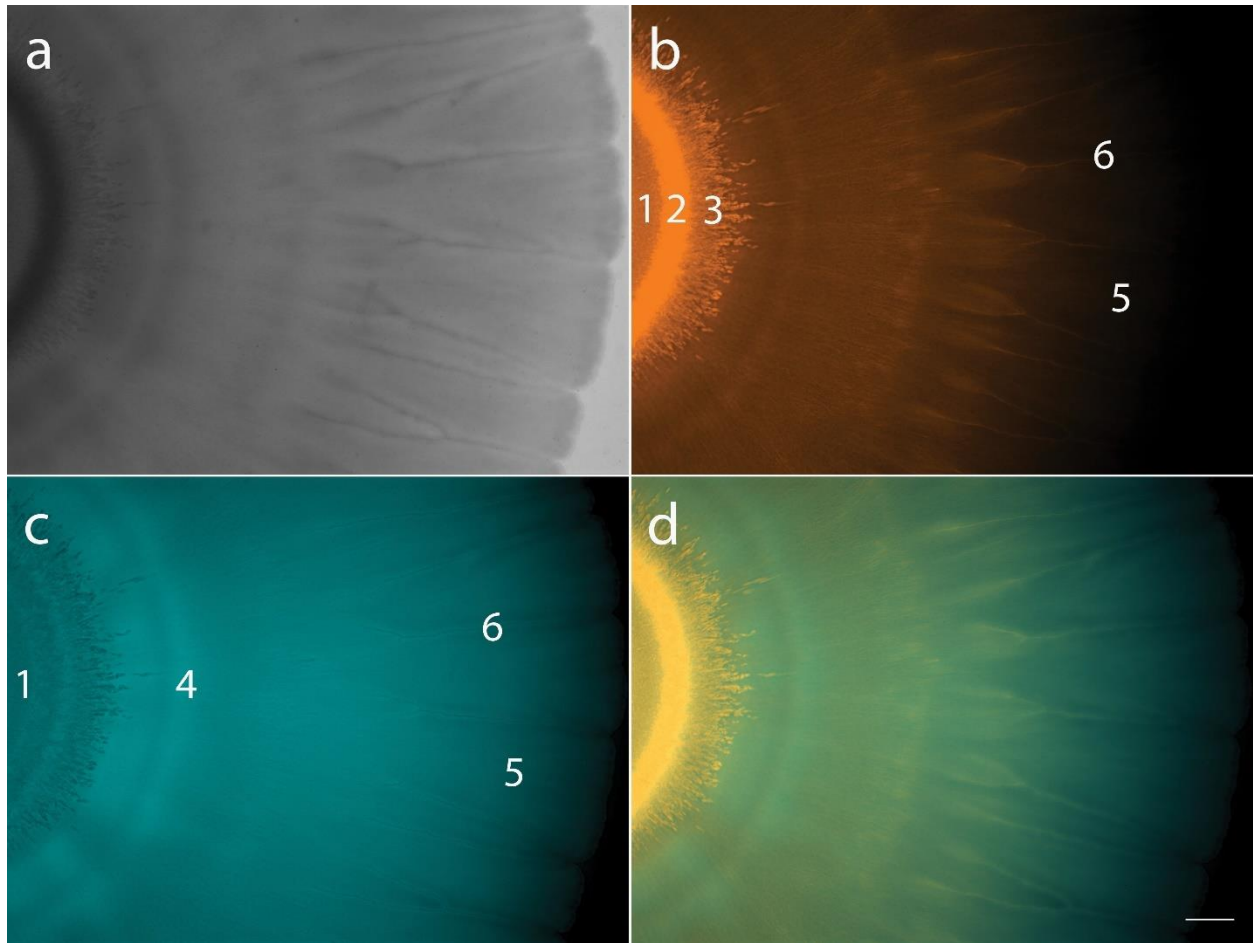
95 The previously observed 'bacterial expansion' in *Pedobacter* when interacting with  
96 *Pseudomonas* sp. AD21 was suggested to be gliding motility, triggered as a mechanism to  
97 escape competition from *P. fluorescens* (de Boer *et al.*, 2007; Garbeva and de Boer, 2009). We  
98 examined the possibility that the motility observed when co-inoculating *Pedobacter* and *P.*  
99 *fluorescens* Pf0-1 was a result of *Pedobacter* moving away from *P. fluorescens*. Bacteria were  
100 collected from the center, middle, and edge of a seven-day-old motile colony. The presence or  
101 absence of each species was tested by culturing these samples on selective media. We  
102 recovered both species from each point in the motile colony (data not shown), showing co-  
103 migration rather than an escape strategy by *Pedobacter*.

## 104 **Social motility shows reproducible spatial organization**

105 To obtain a more detailed look at the spatial relationships within the motile colony, we  
106 tagged *P. fluorescens* with a cyan fluorescent protein (eCFP [Choi and Schweizer, 2006]) and  
107 *Pedobacter* with a red fluorescent protein (dsRedEXPRESS [Choi and Schweizer, 2006]),  
108 integrated into the chromosome. In *P. fluorescens*, eCFP carried by miniTni7 was integrated  
109 upstream of *glmS* (Lambertsen *et al.*, 2004), creating Pf0-*ecfp*. In *Pedobacter*, dsRedEXPRESS  
110 carried by the *HimarEm* transposon (Braun *et al.*, 2005) was integrated at random locations in  
111 the chromosome, resulting in 16 independently-derived mutants with an insert. Each tagged  
112 *Pedobacter* strain (V48-*dsRed*) was indistinguishable from the wild-type in social assays with *P.*  
113 *fluorescens*, indicating no deleterious impact of the insertions. We picked one strain with an  
114 insert in locus N824\_RS25465 (GenBank accession NZ\_AWRU01000034), and no apparent  
115 defect in social motility. The initiation of social motility appeared slightly delayed in a mixture of  
116 the tagged strains, but the visible patterns and stages of development looked identical, and  
117 speed was not significantly different once movement initiated ( $p = 0.0801$ ).

118 Fluorescent microscopy verified culturing data that showed both bacteria are present  
119 throughout the motile colony, but we also found that population density varies across distinct  
120 areas within the colony. These distribution patterns were highly reproducible and show six  
121 distinct zones (Fig. 3). At zone 1, the point of inoculation, fluorescent imaging shows a  
122 homogenous mix of both bacteria (Fig. 3b, c). Zone 2, the coffee ring formed at the edge of the  
123 point of inoculation (Deegan *et al.*, 1997; Sempels *et al.*, 2013; Yanni *et al.*, 2017), is bright  
124 orange, indicating that *Pedobacter* dominates this region (Fig. 3b). *Pedobacter* spreads out from  
125 this dense area into zone 3, in a starburst pattern (Fig. 3b). Just outward from the starburst, we  
126 see a blue ring in multiple experiments (zone 4), where *P. fluorescens* appears more abundant  
127 (Figs. 3c, d). In the main body of the co-culture, a thin motile section spreads out, making  
128 'petals' (Zone 5), with 'veins' (Zone 6) between them (Figs. 2b and 3a). The 'veins' between the  
129 'petals' appear to have high *Pedobacter* populations (Fig. 3b), while the areas directly  
130 surrounding them are dominated by *P. fluorescens* (Fig. 3c). The flat areas of the 'petals'  
131 appear more well-mixed, though the red signal becomes difficult to detect toward the edge of  
132 the colony (Fig. 3d). Overall, imaging data show that we can find both species throughout the  
133 colony, but the distribution is not homogenous. Rather, we observed reproducible patterns with  
134 some well-mixed areas and others of high spatial assortment.





**Figure 3.** Mixed colony of Pf0-*ecfp* and 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.

135 ***Physical association of P. fluorescens Pf0-1 and Pedobacter V48 is required for social***  
136 ***motility***

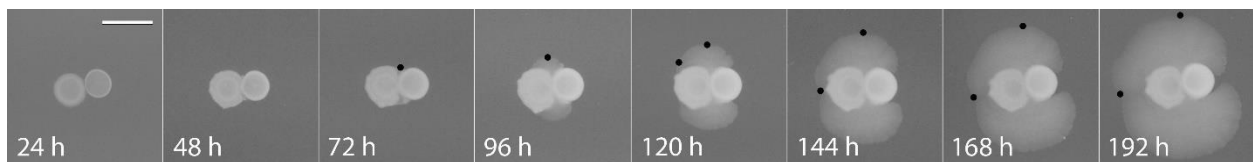
137 Previous studies demonstrated interactions between *P. fluorescens* and *Pedobacter*  
138 were mediated via both diffusible and volatile signals (Garbeva *et al.*, 2011a, 2011b, 2014). We  
139 asked whether a close association between the two bacteria was a necessary condition for  
140 social motility or whether signaling via diffusible compounds could trigger the movement. To  
141 answer this question, we used assays in which the bacterial participants were plated side-by  
142 side with no physical barrier and in which they were separated by semi-permeable membranes.

143 When colonies were adjacent, rather than mixed, no social motility was observed while  
144 the *P. fluorescens* and *Pedobacter* colonies were visibly separate (data not shown). However,



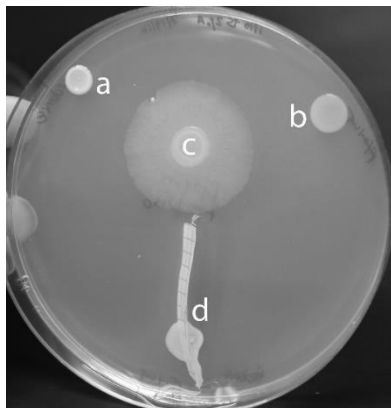
145 once the colonies grew sufficiently to make contact (Fig. 4 24 h), the colony started to spread  
146 out from the point of contact (72 h). The spreading front radiates outward (96 h), first developing  
147 around the *P. fluorescens* colony (144 h), then proceeding to surround the *Pedobacter* colony  
148 (192 h). At this level of resolution, contact between the colonies appears to occur before any  
149 spreading can be seen.

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



**Figure 4.** Social motility emerges after contact between colonies of *P. fluorescens* Pf0-1 (left) and *Pedobacter* sp. V48 (right). 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. Spots indicate sampling locations. Pictures taken every 24 h. Scale bar represents 10 mm.

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

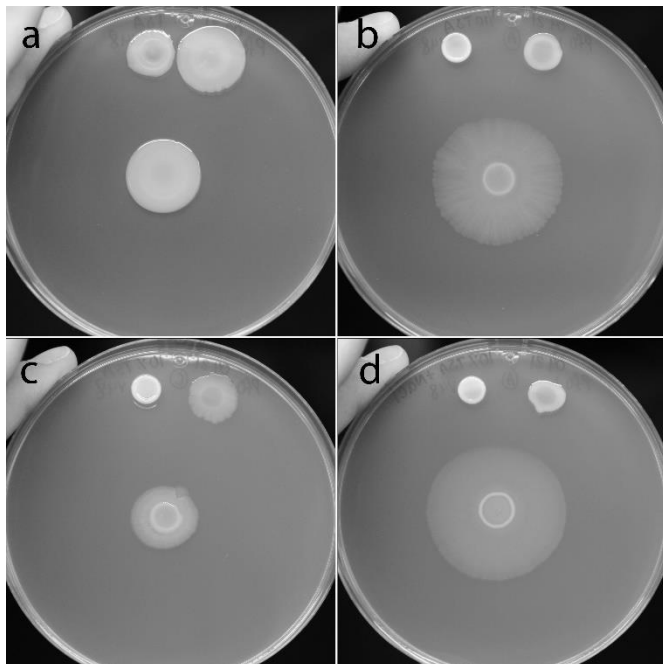


**Figure 5.** A semi-permeable barrier prevents development of the social motility phenotype. a) *Pedobacter* sp. V48 monoculture, b) *P. fluorescens* Pf0-1 monoculture, c) a mixed colony, and d) *P. fluorescens* and *Pedobacter* separated by a mixed-ester cellulose membrane. Pictures taken 144 h after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

163 **Nutritional environment influences social motility**

164 Conditions in soil and rhizosphere environments fluctuate, with bacteria subjected to a  
165 wide range of environmental stressors, including limited nutrient and water availability (Fierer,  
166 2017). Because such fluctuations may influence expression of traits, we examined the effect of  
167 nutrient level on social motility. Our standard assay condition, TSB-NK, consists of 10% strength  
168 Tryptic Soy (3 g/L) supplemented with NaCl (5 g/L) and  $\text{KH}_2\text{PO}_4$  (1 g/L) (Figs. 1b and 6b).

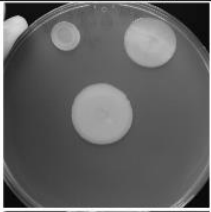

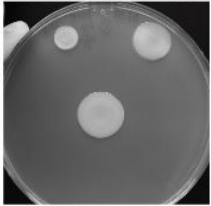
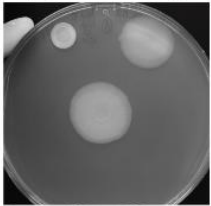
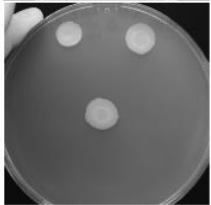
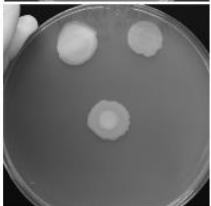
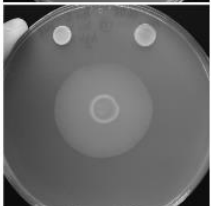
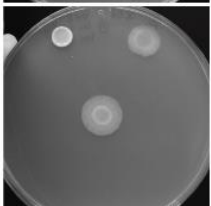
169 We first asked if social motility could initiate under richer nutrient conditions. No social  
170 motility was apparent when *P. fluorescens* and *Pedobacter* were mixed on full-strength TSB (30  
171 g/L) (Fig. 6a), with the co-culture exhibiting the same characteristics and colony expansion as  
172 the *P. fluorescens* mono-culture. We next asked whether the salt amendments to TSB-NK  
173 influence social motility, using assays without the addition of salts, and with the addition of NaCl  
174 and  $\text{KH}_2\text{PO}_4$  individually. When grown on 10% TSB, the co-culture is motile, but the distance  
175 moved is modest compared to when the medium is supplemented with both salts (Fig. 6c). The  
176 individual *P. fluorescens* colony expands similarly to the co-culture, suggesting minimal social  
177 behavior under these conditions. Growth on TSB-K changes neither pattern nor rate of mono-  
178 and co-culture expansion compared to 10% TSB (data not shown). On TSB-N, the mixed  
179 culture spreads and develops the patterns characteristic of social motility, while the *P.*  
180 *fluorescens* mono-culture does not expand (Fig. 6d). The phenotype and diameter of the  
181 spreading colony are most similar to those observed in TSB-NK conditions (Fig. 6b).



**Figure 6.** Low nutrient and high salt conditions are required for social motility. a) Mixed colony on full-strength TSB does not show social motility. b) Mixed colony on TSB-NK (10% Tryptic Soy supplemented with both NaCl and  $\text{KH}_2\text{PO}_4$ ) shows social motility. c) Mixed colony on 10% strength TSB shows impaired social motility. d) Mixed colony on TSB-N (supplemented with NaCl) exhibits the social motility phenotype. For all panels *Pedobacter* sp. V48 mono-culture is on the upper left corner of the plate, *P. fluorescens* Pf0-1 is on the upper right corner 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.

182           In the previous experiment, we observed that variations of Tryptic Soy media led to  
183 altered social phenotypes. To assess the influence of each component of TSB on social motility,  
184 we utilized a medium in which these were individually manipulated. We made eight  
185 combinations of media to vary D-glucose, tryptone, and NaCl in concentrations equivalent to  
186 those in full-strength and 10% TSB. On media with D-glucose or tryptone at full-strength  
187 concentrations, we did not observe social motility regardless of the concentration of the other  
188 components (Figs. 7a-f). In these conditions, the appearance and expansion of the co-culture  
189 resembled that of the *P. fluorescens* mono-culture, with notably greater biomass in media with  
190 full-strength tryptone (Figs. 7a-d). When the concentration of all three components was reduced  
191 to 10% we observed social motility, but the migration distance of the co-culture was modest,  
192 and *P. fluorescens* mono-culture expanded to a similar extent (Fig. 7h). On media containing  
193 10% strength D-glucose, 10% strength tryptone, and full-strength NaCl, social motility emerged  
194 when *P. fluorescens* and *Pedobacter* were co-cultured (Fig. 7g). Unique to this condition, the  
195 mono-cultures of both strains are immotile, indicating a dramatic change in behavior when  
196 strains are mixed. The observations under this condition are most similar to those observed on  
197 TSB-N and TSB-NK (Figs. 6b, d).

198           Based on these results, we conclude that full social motility expansion was only  
199 observed in low nutrient medium supplemented with NaCl (Figs. 6b, d, and 7g). We observed  
200 reduced social motility on low nutrient media without salt supplementation (Figs 6c and 7h), and  
201 an absence of social behavior on rich media (Figs. 6a and 7a-f). While we can implicate salt as  
202 an important factor in social motility, high salt concentrations alone are not sufficient to induce  
203 social behavior, as we don't see social behavior under rich media conditions. This indicates that  
204 there may be more than one important nutritional factor in the decision of these bacteria to  
205 socialize.

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

**Figure 7.** Role of nutrient levels in interaction between *P. fluorescens* 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 upper left corner of the plate, *P. fluorescens* is on the upper right corner 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.

## 221 **Discussion**

222 In this study we investigate social motility, a phenomenon that emerges from the  
223 interaction of two distantly-related soil bacteria. Neither species moves on its own, but a mixture  
224 of the two species can spread across a hard agar surface (2%). Contact between the two  
225 bacterial colonies is required for motility to initiate, and this association is maintained as the co-  
226 culture expands. The social phenotype could be observed only under specific nutritional  
227 conditions, indicating an interplay between environmental and biological factors. Our research  
228 contributes to the growing body of work studying bacteria in social contexts to investigate  
229 emergent traits and behaviors.

230 Surface motility is a trait that could be beneficial to bacteria under a range of  
231 environmental conditions, particularly circumstances which are not permissive for moisture-  
232 dependent translocation. Species related to *Pedobacter* sp. V48 use gliding motility on 1% agar  
233 or glass surfaces (McBride, 2001, 2004; McBride and Zhu, 2013). However, V48 has not been  
234 observed to engage in motility behaviors on its own. *P. fluorescens* Pf0-1 is capable of flagella-  
235 driven swimming in and swarming motility on semi-solid agar (0.3% and 0.6% respectively)  
236 without the need for a partner bacterium (Deflaun *et al.*, 1990; Seaton *et al.*, 2013). Social  
237 motility is distinct from *Pseudomonas* flagellar motility in its requirement of the presence of a  
238 second species. Additionally, media with higher agar percentages form environments that are  
239 non-permissive for flagella-driven motility in *P. fluorescens*, as well as most species, but  
240 together, Pf0-1 and V48 appear to employ an alternative strategy for movement across hard  
241 surfaces.

242 De Boer *et al.* (2007) suggested that in water agar, the sporadic occurrence of  
243 movement they observed indicated a strategy by *Pedobacter* to escape competition. However,  
244 the co-migration under our conditions does not support this hypothesis, as the two species  
245 remain associated throughout the colony. Our contact experiments provide further evidence, as  
246 the presence of *Pedobacter* in the motile areas surrounding the *P. fluorescens* colony shows it  
247 has moved towards its partner, rather than away from it. The pattern of *Pedobacter* migration  
248 clearly indicates that it is not escaping.

249 Evidence, both from culturing and fluorescent imaging, shows that *P. fluorescens* and  
250 *Pedobacter* co-migrate across the hard agar surface. Initiation of the process requires physical  
251 contact, as motility is precluded when a semi-permeable membrane is placed between the two

252 colonies. We suggest that the nature of this interaction is distinct from contact-dependent toxin  
253 delivery systems, such as type VI secretion and contact-dependent growth inhibition, as they  
254 commonly mediate signal exchange between closely-related species, and are involved in  
255 competition between more distantly-related strains (Saak and Gibbs, 2016; Gallique *et al.*, 2017;  
256 Garcia, 2018). While our results do not rule out quorum sensing for communication between the  
257 two species (Juhas *et al.*, 2005), a diffusible signal (if it exists) does not appear to be sufficient to  
258 trigger the motility response. These data indicate that physical association is required for social  
259 motility between *P. fluorescens* and *Pedobacter*.

260 Bacteria dwelling in soil experience variations in a wide range of abiotic conditions,  
261 including the key parameters we have tested: salinity and available carbon and nitrogen  
262 (Reviewed in Fierer, 2017). Environmental conditions have previously been shown to affect  
263 motility of individual species; gliding motility in some *Flavobacterium* species increases with  
264 reduced nutrient concentration (Pérez-Pascual *et al.*, 2009; Laanto *et al.*, 2012). Changes in  
265 behavior resulting from environmental fluctuations can affect how species interact with one  
266 another. The ability of *P. fluorescens* and *Pedobacter* to move socially is dependent upon the  
267 conditions in which they are growing. In general, high concentrations of glucose and amino  
268 acids led to a build-up of biomass and no apparent social movement. Lower glucose and amino  
269 acid concentrations were associated with social motility across the plate, but decreasing the salt  
270 concentration of the media slowed expansion of the colony. Social motility resulting from the  
271 interaction is conditional, with alteration of just a subset of environmental factors resulting in  
272 dramatic changes in behavior. It is tempting to speculate that the consortium of *P. fluorescens*  
273 and *Pedobacter* can integrate signals from each other's presence and from the nutrient  
274 conditions of their environment to determine whether to behave socially. We see similar  
275 examples of intraspecies social behaviors being influenced both by biotic factors (quorum  
276 sensing) and by abiotic factors (nutrient conditions) in *P. aeruginosa* (Boyle *et al.*, 2015),  
277 *Bacillus subtilis* (Lazazzera, 2000), and yeast (Chen and Fink, 2006).

278 There is a wide variety of examples of motility resulting from interspecies interactions,  
279 where the presence of a motile partner fosters the motility of an immotile participant. Non-motile  
280 *Staphylococcus aureus* hitchhikes on swimming *P. aeruginosa* (Samad *et al.*, 2017) and  
281 *Burkholderia cepacia* co-swarms with *P. aeruginosa* in environments where it cannot do so  
282 independently (Venturi *et al.*, 2010). *X. perforans* induces motile *P. vortex* to swarm towards it,  
283 which allows it to hitchhike on top of *P. vortex* rafts (Hagai *et al.*, 2014). *P. vortex* is also capable  
284 of carrying fungal spores or antibiotic-degrading cargo bacteria to cross unfavorable



285 environments (Ingham *et al.*, 2011; Finkelshtein *et al.*, 2015). In an even more complex system,  
286 *Dyella japonica* can migrate on fungal hyphae, but some strains can only do so in the presence  
287 of a *Burkholderia terrae* helper (Warmink and van Elsas, 2009; Warmink *et al.*, 2011). All of  
288 these examples of ‘hitchhiking’ phenomena stand in contrast to the behavior we have  
289 investigated, where social motility emerges from two non-motile participants. The fact that both  
290 species are present at the edge of the moving colony suggests that both have an active role in  
291 the behavior, though it doesn’t rule out the possibility of one species inducing motility in the  
292 other and hitchhiking, as seen in other systems (Hagai *et al.*, 2014).

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

302           We have investigated an interaction-dependent trait which emerges under particular  
303 nutritional conditions when distantly-related bacteria come into close physical contact. This  
304 social motility gives the participating bacteria the ability to spread on a hard agar surface, which  
305 neither can do alone. This strategy of co-migration may serve as an additional mechanism by  
306 which plant- and soil-associated bacteria can move in their natural environments, when the  
307 conditions do not favor the modes of single-species motility previously described. Given the  
308 distant and different locations from which these two strains were isolated, we hypothesize this is  
309 not a unique interaction between this pair, but rather has evolved between various *Pedobacter*  
310 and *Pseudomonas* species. To understand the phenomenon, several lines of investigation  
311 should be pursued: mechanistic studies which explore the factors each species is contributing to  
312 social motility, the process by which contact triggers motility, and the way in which  
313 environmental conditions are integrated into the decision to move together. Such studies will  
314 enable the application of our findings to the search for new examples of interaction-mediated  
315 behaviors among bacteria.

## 316 **Materials and Methods**

317 ***Bacterial strains, primers, plasmids, and culture conditions.*** Bacterial strains and plasmids  
318 are described in Table 1. *E. coli* was grown at 37°C in LB Broth, Miller (Fisher Scientific,  
319 Hampton, NH, U.S.A.). *Pseudomonas fluorescens* Pf0-1 and *Pedobacter* sp. V48 were routinely  
320 grown at 30°C or 20°C respectively, in 10% strength Tryptic Soy Broth (BD Difco™, Franklin  
321 Lakes, NJ, U.S.A.) amended with NaCl and KH<sub>2</sub>PO<sub>4</sub>, as described by de Boer (2007). This  
322 medium is referred to throughout the text as TSB-NK. To differentiate the two species from  
323 mixed cultures we used *Pseudomonas* minimal medium (PMM) with 25 mM succinate (Kirner *et*  
324 *al.*, 1996) for *P. fluorescens* and 14.6 mM lactose for *Pedobacter*. Media were solidified with  
325 1.5% BD Difco™ Bacto™ agar (w/v) when required, except for social motility assays, for which  
326 2% agar was used. For experiments with variations in nutrients, we used full-strength TSB (30  
327 g/L), 10% TSB (3 g/L), and 10% TSB amended with NaCl or KH<sub>2</sub>PO<sub>4</sub> (called TSB-N or TSB-K,  
328 respectively), and a medium composed of D-glucose (2.5 g/L), tryptone (20 g/L), and NaCl (5  
329 g/L). These individual components were used at those concentrations or reduced to 10%  
330 concentration in all eight combinations. For selection of transposon insertions carrying  
331 fluorescent protein genes, Kanamycin (50 µg/mL), Gentamicin (50 µg/mL), or Erythromycin (100  
332 µg/mL) was added to the growth medium.

333 ***Social Motility Assays.*** *P. fluorescens* and *Pedobacter* for use in social motility assays were  
334 incubated in 2mL TSB-NK at 25°C for 24 hours, with shaking (160rpm). Social assays were  
335 carried out on TSB-NK solidified with 2% agar. Plates were poured at a temperature of 62°C in  
336 a single layer and allowed to set for ~15 minutes before inoculation. Inoculation was done on  
337 freshly-poured plates.

338 (i) Mixed inoculum assays. Assays were started by combining 5 µL of each participant in one  
339 spot on the agar surface. As controls, 10µL spots of each bacterial isolate were plated distant  
340 from each other and the co-culture, all on the same plate. Once the inoculation liquid had dried,  
341 plates were incubated at 20°C. Measurements of the colony diameter were taken every 24  
342 hours. Experiments were performed in triplicate.

343 (ii) Direct contact assay - adjacent plating. *P. fluorescens* and *Pedobacter* were grown as  
344 described above. The aliquots of bacteria were plated adjacent but without the drops touching.  
345 Once the inoculation liquid had dried, plates were incubated at 20°C and monitored daily to

346 determine the time at which colony growth led to contact between the isolates, and when  
347 motility phenotypes developed.

348 (iii) Direct contact assay - separation by membranes. *P. fluorescens* and *Pedobacter* were  
349 plated close together, separated only by a membrane. Either Millipore Polyethersulfone (PES)  
350 Express Plus® Membrane (0.22µm pores) (Darmstadt, Germany), or Gelman Sciences mixed-  
351 ester cellulose Metrical Membrane (0.45µm pores) (East Lansing, MI, U.S.A.) were cut into  
352 rectangular strips and sterilized by autoclaving. These strips were then embedded into the agar  
353 by suspending them perpendicular to the bottom of petri dishes with forceps, as agar was  
354 poured into plates. Once set, the filters protruded approximately 5mm above the agar surface.  
355 Bacteria were inoculated on either side of the filter, with 5µL spots of each species, close  
356 enough to touch the filter.

### 357 **Fluorescent protein tagging**

358 (i) eCFP labeling of *P. fluorescens*. pUC18T-mini-Tn7T-Gm-*ecfp* was a gift from Herbert  
359 Schweizer (Addgene plasmid # 65030). A constitutively-expressed fluorescent protein gene  
360 carried by pUC18T-mini-Tn7T-Gm-*ecfp* was transferred to *P. fluorescens* by conjugation from *E.*  
361 *coli* S17-1, with transposase being provided by pUX-BF13 introduced from a second *E. coli*  
362 S17-1 donor, as previously described (Monds *et al.*, 2006). Transposon-carrying strains were  
363 selected by growth on Gentamicin (50 µg/mL), and transposition of the miniTn7 element into the  
364 target site in the *P. fluorescens* genome was confirmed by PCR using primers Tn7-F and *glmS*-  
365 R (Table 2). Pf0-1 with fluorescent inserts were tested for alteration in social motility by co-  
366 culturing with *Pedobacter*, as described above.

367 (ii) dsRedEXPRESS labeling *Pedobacter*. pUC18T-mini-Tn7T-Gm-*dsRedExpress* was a gift  
368 from Herbert Schweizer (Addgene plasmid #65032). To express *dsRedEXPRESS* in  
369 *Pedobacter*, a *Pedobacter* promoter was cloned upstream of the *dsRedEXPRESS* coding  
370 sequence. A highly expressed gene from an unpublished RNAseq experiment was identified  
371 (N824\_RS25200) and the upstream 320 bp were amplified from *Pedobacter* genomic DNA  
372 using primers *PompA* and *dsRed*, designed for splicing-by-overlap extension-PCR (SOE-PCR)  
373 (Table 2). The promoter was then spliced with the amplified *dsRedEXPRESS* coding sequence  
374 using SOE-PCR (Horton *et al.*, 1989). Flanking primers were designed with *KpnI* restriction  
375 sites, enabling cloning of the spliced product into a *KpnI* site in *pHimarEm1* (Braun *et al.*, 2005).  
376 To join compatible ends between the plasmid and the amplicons, we used T4 DNA ligase (New

377 England Biolabs, Inc. Ipswich, MA, U.S.A.). The ligated plasmid was introduced into *E. coli* S17-  
378 1 competent cells by electroporation (BioRad Micropulser™, Hercules, CA, U.S.A.). S17-1  
379 colonies carrying the plasmid were selected by plating on LB medium containing Kanamycin (50  
380 µg/mL), and the presence of the *dsRedEXPRESS* gene was confirmed by PCR, using *pHimar*  
381 *KpnI*-flank primers (Table 2). The resulting plasmid is called *pHimarEm1-dsRed*.

382 *pHimarEm1-dsRed* was transferred to *Pedobacter* by conjugation using a method adapted from  
383 Hunnicutt and McBride, 2000. Briefly, 20 hour old cultures of *E. coli* S17-1 (*pHimarEm1-dsRed*)  
384 and *Pedobacter* were subcultured 1:100 into fresh LB, and grown to mid-exponential phase (*E.*  
385 *coli*) or for 7 hours (*Pedobacter*). Cells were collected by centrifugation, suspended in 100µL of  
386 LB, and then mixed in equal amounts on TSB-NK with 100 µL of 1M CaCl<sub>2</sub> spread on the  
387 surface. Following overnight incubation at 30°C, cells were scraped off the surface of the plate,  
388 and dilutions were plated on TSB-NK with Erythromycin (100 µg/mL) to select for strains that  
389 received the plasmid (*ermF* is not expressed in *E. coli*). Transconjugants were incubated at  
390 25°C for 3-4 days. Presence of the transposon in *Pedobacter* was confirmed using *ermF*  
391 primers (Table 2).

392 The transposon insertion sites in the *Pedobacter* chromosome were amplified by arbitrarily-  
393 primed PCR (Caetano-Anollés, 1993), using a method adapted from O'Toole *et al.*, 1999 (see  
394 table 2 for primers), and identified by sequencing the arb-PCR products. Nucleic acid  
395 sequencing was performed by Massachusetts General Hospital CCIB DNA Core (Cambridge,  
396 MA, U.S.A.). Sequences were analyzed using CLC Genomics Workbench Version 10.1.1  
397 (QIAGEN, Hilden, Germany) to find location of transposon integration.

### 398 **Imaging**

399 Still pictures were taken using an EOS Rebel T3i camera (Canon, Tokyo, Japan) and processed  
400 using Photoshop CC 2017 Version: 2017.0.1 20161130.r.29 (Adobe, San Jose, CA, U.S.A.).  
401 Using this software, the levels of some images were adjusted to improve contrast, and pictures  
402 were converted to greyscale.

403 For microscopy, motile colonies were examined using an Axio Zoom.V16 microscope (Zeiss,  
404 Oberkochen, Germany). To visualize fluorescent strains, filter set 43 HE DsRed was used with a  
405 1.5 s exposure, shown with pseudo-color orange, as well as filter set 47 HE Cyan Fluorescent  
406 Protein, with a 600 ms exposure, shown with pseudo-color turquoise. Images were captured

407 using Axiocam 503 mono camera, with a native resolution of 1936x1460 pixels. For image  
408 acquisition and processing we used Zen 2 Pro software (Zeiss).

#### 409 **Statistics**

410 We measured the amount of colony expansion of the mono-cultures of both *P. fluorescens* and  
411 *Pedobacter* and the expansion of social motility in co-culture. Colony diameter of three  
412 independent experiments was measured every 24 hours. To compare the diameter of mono-  
413 cultures and co-cultures at each time point, we performed a two-way ANOVA followed by a  
414 Bonferroni post-hoc test.

415 We compared the movement speed between a combination of wild type *P. fluorescens* and  
416 *Pedobacter* to a combination of fluorescently-tagged Pf0-ecfp and V48-dsRed. Colony diameter  
417 of six independent experiments were measured every day, and speed was calculated by  
418 dividing the distance traveled by the amount of time elapsed since the last time point. To  
419 calculate average speed, we only used time points after social motility phenotype developed. To  
420 compare the means of the speed of the wild-type and tagged strains, we conducted an  
421 unpaired, two-tailed, Student's t-test.

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

<b>Strain</b>	<b>Genotype or Description</b>	<b>Source or Reference</b>
<b><i>E. coli</i></b>		
S17-1	<i>recA thi pro hsdR</i> RP4-2-Tc::Mu-Km::Tn7 $\lambda$ pir Sm <sup>r</sup> Tp <sup>r</sup>	Simon, Priefer and Puhler, 1983
<b><i>P. fluorescens</i></b>		
Pf0-1	Wild type, Ap <sup>r</sup>	Compeau <i>et al.</i> , 1988
Pf0- <i>ecfp</i>	Pf0-1::mini-Tn7 <i>ecfp</i> Gm <sup>r</sup>	This study
<b><i>Pedobacter</i></b>		
V48	Wild type	De Boer <i>et al.</i> , 2003
V48- <i>dsRed</i>	V48 N824_RS25465T899::HimarEm1 <i>PompA-dsRed</i> EXPRESS Em <sup>r</sup> NCBI Accession: NZ_AWRU00000000.1, as of September 25, 2017	This study
<b>Plasmids</b>		
pUC18T-mini-Tn7T-Gm- <i>dsRed</i> EXPRESS	Gm <sup>r</sup>	Choi & Schweizer, 2006
pUC18T-mini-Tn7T-Gm- <i>ecfp</i>	Gm <sup>r</sup>	Choi & Schweizer, 2006
pUX-BF13	R6K replicon-based helper plasmid carrying Tn7 transposase genes	Bao <i>et al.</i> , 1991
pHimarEm1	Plasmid carrying <i>HimarEm1</i> ; Km <sup>r</sup> (Em <sup>r</sup> )	Braun <i>et al.</i> , 2005
pHimarEm1- <i>dsRed</i>	pHimarEm1 $\Omega$ (3.529kb::PompA- <i>dsRed</i> EXPRESS)	This study

427

428 **Table 2. Primers**

<b>Primers</b>	<b>Sequence (5'-3')</b>	<b>Purpose</b>	<b>Source or Reference</b>
Tn7 F	5'-CAGCATAACTGGACTGATTTAG - 3'	Verify integration of transposon into chromosomal <i>glmS</i> locus	Monds <i>et al.</i> , 2006
<i>glmS</i> R	5'-TGCTCAAGGGCACTGACG-3'	"	Monds <i>et al.</i> , 2006
<i>PompA-dsRed</i> F	5'ACGTTCTCGGAGGAGGCCATCAAC GCAACAAAAGAACTGC 3'	Amplification of N824_RS25200 promoter to join with <i>dsRed</i> gene	This study
<i>PompA</i> R	5'-TATGGTACC AGTCATCTAGCGGCTGTAG-3'	" Includes KpnI-site for inserting into pHimarEm1	This study
<i>dsRed</i> F	5'-TACTCAGGAGAGCGTTCACC-3'	Amplification of <i>dsRed</i> gene with no promoter, to join with V48 N824_RS25200 promoter by SOE PCR	This study
<i>dsRed</i> R	5'- GCAGTTTCTTTTGTTCGTTGATGGC CTCCTCCGAGAACGT-3'	" Includes KpnI-site for inserting into pHimarEm1	This study
pHimar KpnI-flank F	5'-CTGCCCTGCAATCGACCTCG-3'	Verify ligation of <i>dsRed</i> into pHimarEm1	This study
pHimar KpnI-flank R	5'-CAGATAGCCCAGTAGCTGAC-3'	"	This study
<i>erm</i> F	5'-CCGCACCCAAAAAGTTGCAT-3'	Verify integration of transposon into V48 chromosome.	This study
<i>erm</i> R	5'-GACAATGGAACCTCCCAGAA-3'	"	This study
ARB1	5'- GGCCACGCGTCGACTAGTACNNNNN NNNNNGATAT-3'	Find location of transposon integration in V48 chromosome.	O'Toole <i>et al.</i> , 1999
ARB6	5'- GGCCACGCGTCGACTAGTACNNNNN NNNNNACGCC-3'	"	O'Toole <i>et al.</i> , 1999
ARB2	5'-GGCCACGCGTCGACTAGTAC -3'	"	O'Toole <i>et al.</i> , 1999
Himar Arb1 (TnExt)	5'-GTGTTGTTCCAGTTTGAGATC-3'	"	This study
Himar609 Arb2 (TnInt)	5'-TGGGAATCATTGAAGTTGG-3'	"	Braun <i>et al.</i> , 2005

429

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