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

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Running title: Social motility as an emergent trait

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Abstract

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

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Introduction

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

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

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

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

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

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Results

Social motility arises when mixing two distantly-related bacteria.

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

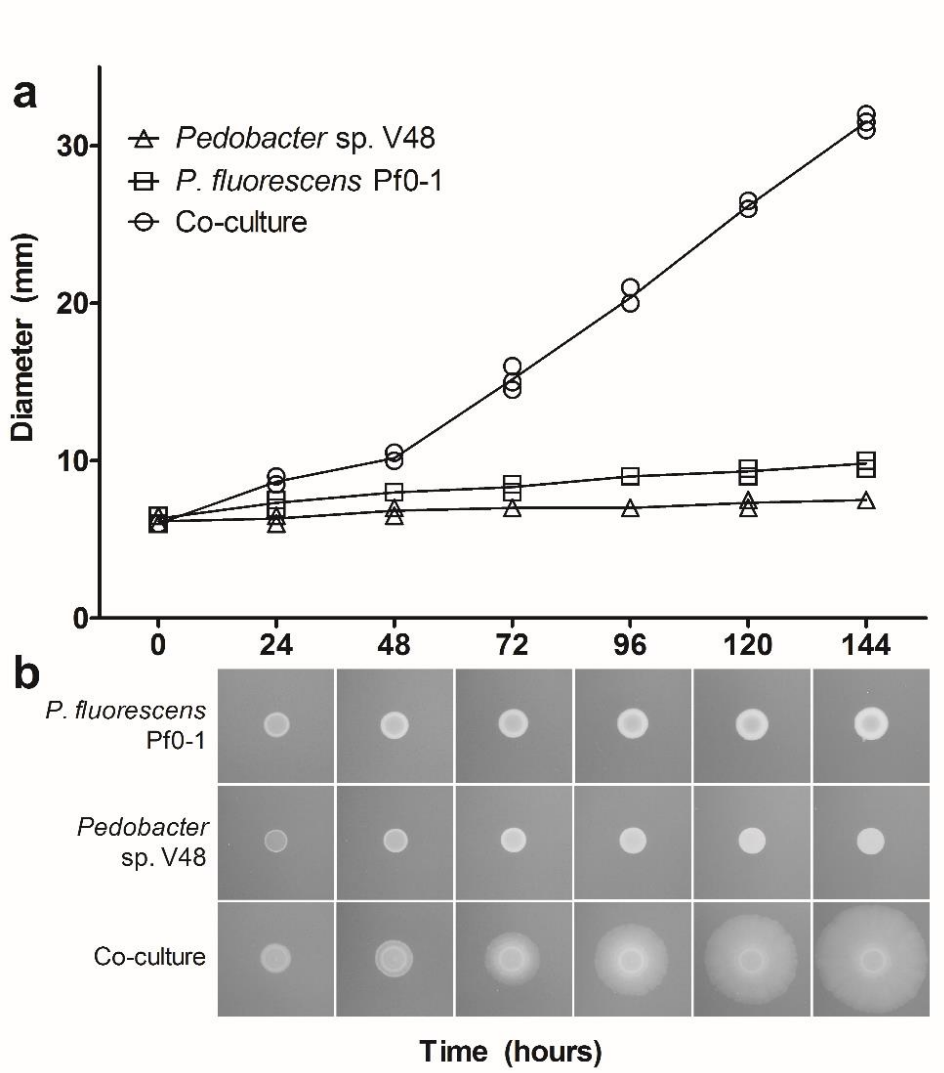

Figure 1. Mixed colony of *P. fluorescens* Pf0-1 and *Pedobacter* sp. V48 spreads across a hard agar surface (2%), a behavior not observed in the mono-culture of either species. a) Diameter of colonies at 24 h intervals. b) Phenotypes of mono- and co-cultures at 24 h intervals. Contrast and brightness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

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

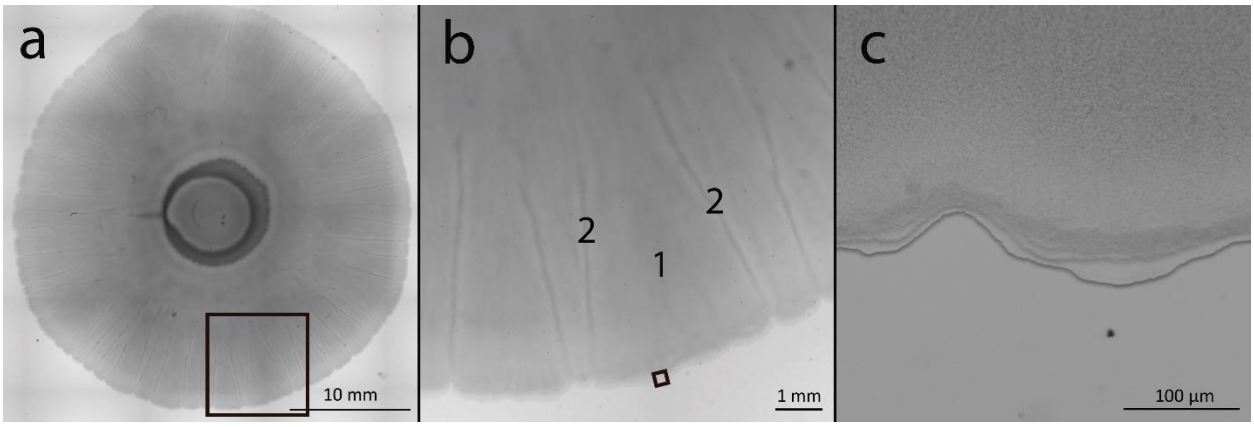

Figure 2. Mixed colony of *P. fluorescens* Pf0-1 and *Pedobacter* sp. V48 at different magnifications. a) Image of the whole co-culture colony created by stitching an 8X magnification mosaic. b) 16X magnification of the leading edge showing the patterns of 'petals' (1) in between 'veins' (2) visible 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.

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

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

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Social motility shows reproducible spatial organization

To obtain a more detailed look at the spatial relationships within the motile colony, we tagged *P. fluorescens* with a cyan fluorescent protein (eCFP [Choi and Schweizer, 2006]) and *Pedobacter* with a red fluorescent protein (dsRedEXPRESS [Choi and Schweizer, 2006]), integrated into the chromosome. In *P. fluorescens*, eCFP carried by miniTni*7* was integrated upstream of *glmS* (Lambertsen *et al.*, 2004), creating Pf0-*ecfp*. In *Pedobacter*, dsRedEXPRESS carried by the *HimarEm* transposon (Braun *et al.*, 2005) was integrated at random locations in the chromosome, resulting in 16 independently-derived mutants with an insert. Each tagged *Pedobacter* strain (V48-*dsRed)* was indistinguishable from the wild-type in social assays with *P. fluorescens*, indicating no deleterious impact of the insertions. We picked one strain with an insert in locus N824_RS25465 (GenBank accession NZ_AWRU01000034), and no apparent defect in social motility. The initiation of social motility appeared slightly delayed in a mixture of the tagged strains, but the visible patterns and stages of development looked identical, and speed was not significantly different once movement initiated ($p = 0.0801$).

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

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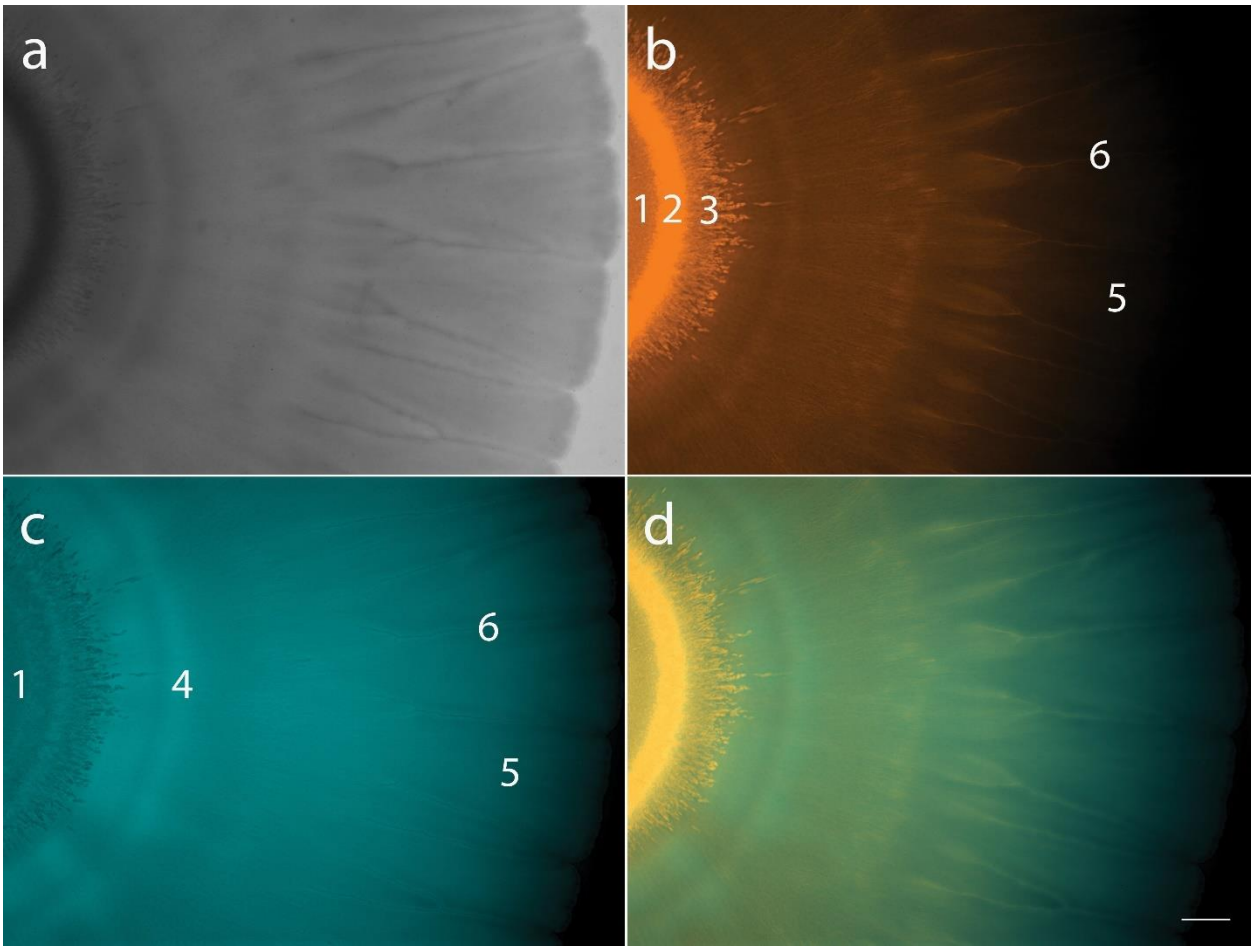

Figure 3. Mixed colony of Pf0-*ecfp* and V48-*dsRed*. a) Co-culture colony viewed with white light. b) Co-culture imaged using DsRed filter (filter set 43 HE), pseudo-colored in orange, showing V48-*dsRed* distribution throughout the colony. c) Co-culture imaged using CFP filter (filter set 47 HE), pseudo-colored 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.

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

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

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

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

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

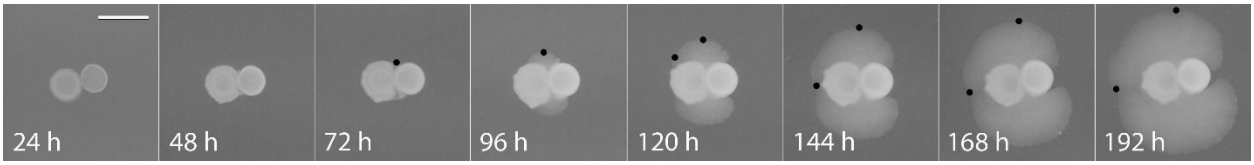

Figure 4. Social motility emerges after contact between colonies of *P. fluorescen*s Pf0-1 (left) and *Pedobacter* sp. V48 (right). Colonies come into contact 24 hours after inoculation; the motile front becomes visible 48 hours after contact and spreads outward and around the *P. fluorescens* colony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

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

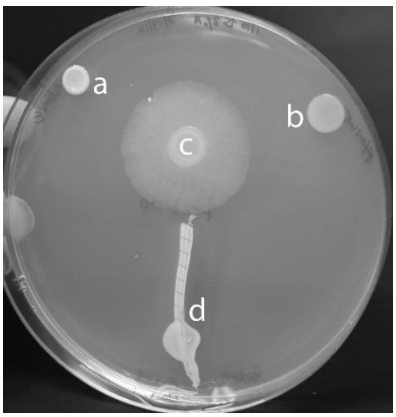

Figure 5. A semi-permeable barrier prevents development of the social motility phenotype. a) *Pedobacter* sp. V48 monoculture, b) *P. fluorescens* Pf0-1 monoculture, c) a mixed colony, and d) *P. fluorescens* and *Pedobacter* separated by a mixed-ester cellulose membrane. Pictures taken 144 h after inoculation.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

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Nutritional environment influences social motility

Conditions in soil and rhizosphere environments fluctuate, with bacteria subjected to a wide range of environmental stressors, including limited nutrient and water availability (Fierer, 2017). Because such fluctuations may influence expression of traits, we examined the effect of nutrient level on social motility. Our standard assay condition, TSB-NK, consists of 10% strength Tryptic Soy (3 g/L) supplemented with NaCl (5 g/L) and KH_2PO_4 (1 g/L) (Figs. 1b and 6b).

We first asked if social motility could initiate under richer nutrient conditions. No social motility was apparent when *P. fluorescens* and *Pedobacter* were mixed on full-strength TSB (30 g/L) (Fig. 6a), with the co-culture exhibiting the same characteristics and colony expansion as the *P. fluorescens* mono-culture. We next asked whether the salt amendments to TSB-NK influence social motility, using assays without the addition of salts, and with the addition of NaCl and KH_2PO_4 individually. When grown on 10% TSB, the co-culture is motile, but the distance moved is modest compared to when the medium is supplemented with both salts (Fig. 6c). The individual *P. fluorescens* colony expands similarly to the co-culture, suggesting minimal social behavior under these conditions. Growth on TSB-K changes neither pattern nor rate of mono- and co-culture expansion compared to 10% TSB (data not shown). On TSB-N, the mixed culture spreads and develops the patterns characteristic of social motility, while the *P. fluorescens* mono-culture does not expand (Fig. 6d). The phenotype and diameter of the spreading colony are most similar to those observed in TSB-NK conditions (Fig. 6b).

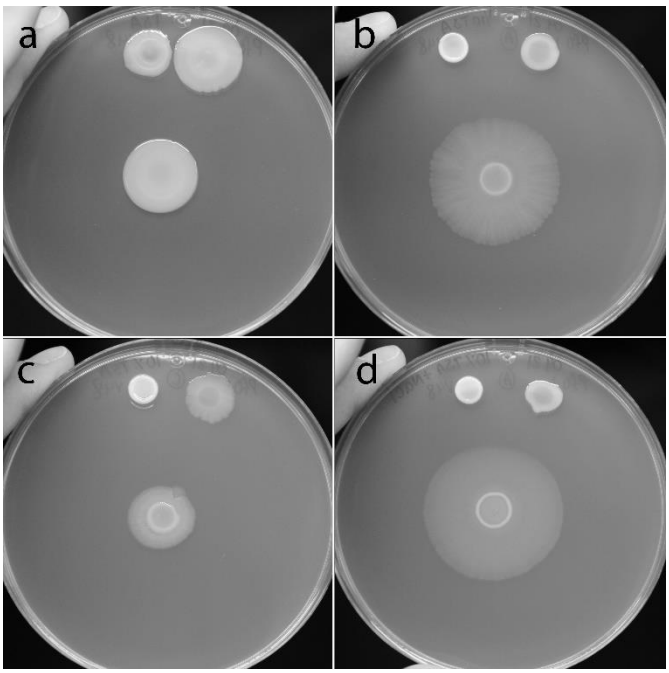

Figure 6. Low nutrient and high salt conditions are required for social motility. a) Mixed colony on full-strength TSB does not show social motility. b) Mixed colony on TSB-NK (10% Tryptic Soy supplemented with both NaCl and KH_2PO_4) shows social motility. c) Mixed colony on 10% strength TSB shows impaired 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.

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

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

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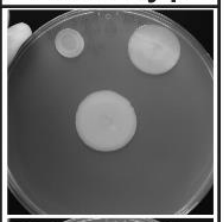
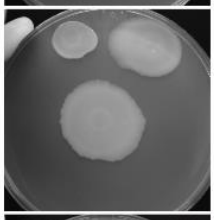
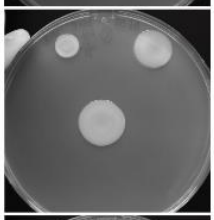
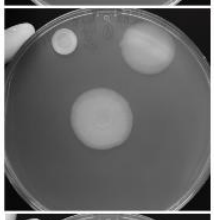
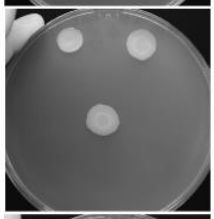
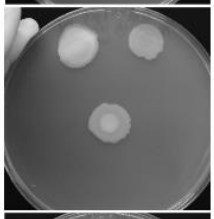
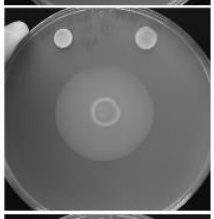
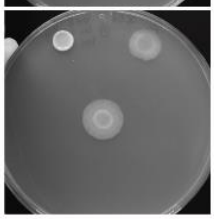
Glucose	Tryptone	NaCl	Phenotype
a High	High	High	
b High	High	Low	
c Low	High	High	
d Low	High	Low	
e High	Low	High	
f High	Low	Low	
g Low	Low	High	
h Low	Low	Low	

Figure 7. 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' conentrations. 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.

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Discussion

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

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

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

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

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

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

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

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

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

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

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Materials and Methods

Bacterial strains, primers, plasmids, and culture conditions. Bacterial strains and plasmids are described in Table 1. *E. coli* was grown at 37°C in LB Broth, Miller (Fisher Scientific, Hampton, NH, U.S.A.). *Pseudomonas fluorescens* Pf0-1 and *Pedobacter* sp. V48 were routinely grown at 30°C or 20°C respectively, in 10% strength Tryptic Soy Broth (BD Difco™, Franklin Lakes, NJ, U.S.A.) amended with NaCl and KH_2PO_4, as described by de Boer (2007). This medium is referred to throughout the text as TSB-NK. To differentiate the two species from mixed cultures we used *Pseudomonas* minimal medium (PMM) with 25 mM succinate (Kirner *et al.*, 1996) for *P. fluorescens* and 14.6 mM lactose for *Pedobacter*. Media were solidified with 1.5% BD Difco™ Bacto™ agar (w/v) when required, except for social motility assays, for which 2% agar was used. For experiments with variations in nutrients, we used full-strength TSB (30 g/L), 10% TSB (3 g/L), and 10% TSB amended with NaCl or KH_2PO_4 (called TSB-N or TSB-K, respectively), and a medium composed of D-glucose (2.5 g/L), tryptone (20 g/L), and NaCl (5 g/L). These individual components were used at those concentrations or reduced to 10% concentration in all eight combinations. For selection of transposon insertions carrying fluorescent protein genes, Kanamycin (50 µg/mL), Gentamicin (50 µg/mL), or Erythromycin (100 µg/mL) was added to the growth medium.

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

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

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

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

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

Fluorescent protein tagging

(i) eCFP labeling of *P. fluorescens*. pUC18T-mini-Tn*7*T-Gm-*ecfp* was a gift from Herbert Schweizer (Addgene plasmid # 65030). A constitutively-expressed fluorescent protein gene carried by pUC18T-mini-Tn*7*T-Gm-*ecfp* was transferred to *P. fluorescens* by conjugation from *E. coli* S17-1, with transposase being provided by pUX-BF13 introduced from a second *E. coli* S17-1 donor, as previously described (Monds *et al.*, 2006). Transposon-carrying strains were selected by growth on Gentamicin (50 µg/mL), and transposition of the miniTn*7* element into the target site in the *P. fluorescens* genome was confirmed by PCR using primers Tn*7*-F and *glmS*-R (Table 2). Pf0-1 with fluorescent inserts were tested for alteration in social motility by co-culturing with *Pedobacter*, as described above.

(ii) dsRedEXPRESS labeling *Pedobacter*. pUC18T-mini-Tn*7*T-Gm-*dsRedExpress* was a gift from Herbert Schweizer (Addgene plasmid #65032). To express *dsRedEXPRESS* in *Pedobacter*, a *Pedobacter* promoter was cloned upstream of the *dsRedEXPRESS* coding sequence. A highly expressed gene from an unpublished RNAseq experiment was identified (N824_RS25200) and the upstream 320 bp were amplified from *Pedobacter* genomic DNA using primers P*ompA* and *dsRed*, designed for splicing-by-overlap extension-PCR (SOE-PCR) (Table 2). The promoter was then spliced with the amplified *dsRedEXPRESS* coding sequence using SOE-PCR (Horton *et al.*, 1989). Flanking primers were designed with *Kpn*I restriction sites, enabling cloning of the spliced product into a *Kpn*I site in *pHimarEm1* (Braun *et al.*, 2005). To join compatible ends between the plasmid and the amplicons, we used T4 DNA ligase (New

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

pHimarEm1-dsRed was transferred to *Pedobacter* by conjugation using a method adapted from Hunnicutt and McBride, 2000. Briefly, 20 hour old cultures of *E. coli* S17-1 (*pHimarEm1-dsRed*) and *Pedobacter* were subcultured 1:100 into fresh LB, and grown to mid-exponential phase (*E. coli*) or for 7 hours (*Pedobacter*). Cells were collected by centrifugation, suspended in 100µL of LB, and then mixed in equal amounts on TSB-NK with 100 µL of 1M $CaCl_2$ spread on the surface. Following overnight incubation at 30°C, cells were scraped off the surface of the plate, and dilutions were plated on TSB-NK with Erythromycin (100 µg/mL) to select for strains that received the plasmid (*ermF* is not expressed in *E. coli*). Transconjugants were incubated at 25°C for 3-4 days. Presence of the transposon in *Pedobacter* was confirmed using *ermF* primers (Table 2).

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

Imaging

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

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

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

Statistics

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

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

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426 **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	Simon, Priefer and Puhler, 1983
P. fluorescens		
Pf0-1	Wild type, Ap^r	Compeau *et al.*, 1988
Pf0-*ecfp*	Pf0-1::mini-Tn*7 ecfp* Gm^r	This study
Pedobacter		
V48	Wild type	De Boer *et al.*, 2003
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	Choi & Schweizer, 2006
pUC18T-mini-Tn7T-Gm-ecfp	Gm^r	Choi & Schweizer, 2006
pUX-BF13	R6K replicon-based helper plasmid carrying Tn*7* transposase genes	Bao *et al.*, 1991
pHimarEm1	Plasmid carrying *HimarEm1*; Km^r (Em^r)	Braun *et al.*, 2005
pHimarEm1-dsRed	pHimarEm1Ω(3.529kb::P*ompA-dsRedEXPRESS)*	This study

427

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428 **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	Monds *et al.*, 2006
glmS R	5'-TGCTCAAGGGCACTGACG-3'	"	Monds *et al.*, 2006
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.	O'Toole *et al.*, 1999
ARB6	5'-GGCCACGCGTCGACTAGTACNNNNN NNNNNACGCC-3'	"	O'Toole *et al.*, 1999
ARB2	5'-GGCCACGCGTCGACTAGTAC -3'	"	O'Toole *et al.*, 1999
Himar Arb1 (TnExt)	5'-GTGTTGTTCCAGTTTGAGATC-3'	"	This study
Himar609 Arb2 (TnInt)	5'-TGGGAATCATTTGAAGGTTGG-3'	"	Braun *et al.*, 2005

429

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