



Rhizobial Chemotaxis and Motility Systems at Work in the Soil

Samuel T. N. Aroney, Philip S. Poole and Carmen Sánchez-Cañizares*

Department of Plant Sciences, University of Oxford, Oxford, United Kingdom

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*Correspondence:

Carmen Sánchez-Cañizares
carmen.sanchez-canizares@
plants.ox.ac.uk

Specialty section:

This article was submitted to
Plant Symbiotic Interactions,
a section of the journal
Frontiers in Plant Science

Received: 15 June 2021

Accepted: 06 August 2021

Published: 27 August 2021

Citation:

Aroney STN, Poole PS and
Sánchez-Cañizares C (2021) Rhizobial
Chemotaxis and Motility Systems at
Work in the Soil.
Front. Plant Sci. 12:725338.
doi: 10.3389/fpls.2021.725338

Bacteria navigate their way often as individual cells through their chemical and biological environment in aqueous medium or across solid surfaces. They swim when starved or in response to physical and chemical stimuli. Flagella-driven chemotaxis in bacteria has emerged as a paradigm for both signal transduction and cellular decision-making. By altering motility, bacteria swim toward nutrient-rich environments, movement modulated by their chemotaxis systems with the addition of pili for surface movement. The numbers and types of chemoreceptors reflect the bacterial niche and lifestyle, with those adapted to complex environments having diverse metabolic capabilities, encoding far more chemoreceptors in their genomes. The Alpha-proteobacteria typify the latter case, with soil bacteria such as rhizobia, endosymbionts of legume plants, where motility and chemotaxis are essential for competitive symbiosis initiation, among other processes. This review describes the current knowledge of motility and chemotaxis in six model soil bacteria: *Sinorhizobium meliloti*, *Agrobacterium fabacearum*, *Rhizobium leguminosarum*, *Azorhizobium caulinodans*, *Azospirillum brasilense*, and *Bradyrhizobium diazoefficiens*. Although motility and chemotaxis systems have a conserved core, rhizobia possess several modifications that optimize their movements in soil and root surface environments. The soil provides a unique challenge for microbial mobility, since water pathways through particles are not always continuous, especially in drier conditions. The effectiveness of symbiont inoculants in a field context relies on their mobility and dispersal through the soil, often assisted by water percolation or macroorganism movement or networks. Thus, this review summarizes the factors that make it essential to consider and test rhizobial motility and chemotaxis for any potential inoculant.

Keywords: rhizobia, *Rhizobium leguminosarum*, *Sinorhizobium meliloti*, *Azospirillum brasilense*, *Bradyrhizobium diazoefficiens*, motility, chemotaxis, soil

INTRODUCTION

There are several strategies bacteria use to actively navigate their environment, motile forces produced either through pili retraction or flagella rotation. Pili drive twitching motility across surfaces by extending, adhering and retracting to pull the bacterium forward (Mattick, 2002), whereas flagella are rigid helical structures, anchored to the cell wall and rotated by a protein motor to produce thrust (Macnab and Aizawa, 1984). This enables the bacteria to move sporadically as individuals (swimming) or continuously as an organized group (swarming) (reviewed by Henrichsen, 1972). Alternatively, organized and continuous movement can be driven by motor proteins anchored to a surface or nearby bacterium, moving along helical tracks in the inner membrane and pushing the cell forwards (gliding) (Nan and Zusman, 2016). Other, passive

strategies have also been described that are driven by the expansion of a growing culture, with the bacteria either producing substances to reduce friction and enable mass movement (sliding) or producing an aggregate capsule from which cells are ejected (darting) (Henrichsen, 1972; Pollitt and Diggle, 2017).

Active bacterial motility tends to be controlled by chemotaxis systems that respond to different stimuli, allowing bacteria to migrate to optimal environments. This occurs through sensing the binding of a ligand to its cognate chemoreceptors (methyl accepting chemotaxis proteins, MCPs). In response to signal transduction, motility systems produce runs, tumbles, reverses, pauses and other phenomena that together form a biased three-dimensional walk. Although tumbles, reverses and pauses are random reorientation events, the movement of bacteria is biased by controlling the frequency of these events. Some bacterial flagella rotate bidirectionally, others unidirectionally, to bias stopping and starting (Kearns, 2010).

An impressive diversity of motility mechanisms has evolved in prokaryotes. Among Gram-negative bacteria, *Escherichia coli* is the best understood model of flagella-based swimming. Also belonging to this group are a variety of nitrogen-fixing soil bacteria, known as rhizobia, that have evolved several differences from the *E. coli* model and are compared in this review. These include *Sinorhizobium meliloti* RU11 (also *Ensifer meliloti*), which nodulates alfalfa (*Medicago sativa*) and *Medicago truncatula* (Meade et al., 1982); *Agrobacterium fabacearum* H13-3 (formerly *R. lupini* H13-3), which nodulates *Lupinus luteus*, the European yellow lupin (Delamuta et al., 2020); *Rhizobium leguminosarum* bv. *viciae* 3841, which nodulates pea (*Pisum sativum*), various *Vicia*, lentils (*Lens culinaris*), grass peas and sweet peas (various *Lathyrus*) (Young et al., 2006); *Azorhizobium caulinodans* ORS571, which nodulates *Sesbania rostrata* (Lee et al., 2008); *Azospirillum brasilense* Sp7, a non-endosymbiote but a microaerobic diazotroph that colonizes the rhizospheres of grasses (Zhulin and Armitage, 1993); and finally, *Bradyrhizobium diazoefficiens* USDA110 (formerly *B. japonicum*), which nodulates soybean (*Glycine max*) (Kaneko et al., 2002). The relevance of motility and chemotaxis for rhizobia, both as free-living bacteria in the soil and as symbiotic cells inside plant nodules, is then discussed in the context of rhizobial inoculants and the importance of evaluating the chemotaxis and motility properties of strains used in the field.

The *Escherichia coli* Motility Model and Marine Bacteria Motility

E. coli typically have 5–10 flagella protruding from various points around the cell body (peritrichous flagellation) that form an integrated bundle oriented in the same direction (Macnab and Aizawa, 1984). Each flagellum consists of a basal body, hook and filament (see **Figure 1A**). The basal body is made up of a central hollow rod surrounded by anchoring stacks of rings (Wang et al., 2012). At the base of the basal body is the motor, where torque is produced via transmembrane gradients of H⁺ ions, and in other bacterial species, also of Na⁺ or K⁺ ions (Hirota

and Imae, 1983; Armitage and Schmitt, 1997; Kojima and Blair, 2001; Terahara et al., 2012). This motor is further subdivided into the rotor, the proteins that rotate with the flagella, and the stator, the proteins driving rotation. The hook is a highly curved helix; a flexible coupling that connects the central rod to the filament. The filament is a left-handed helix consisting of a single protein, flagellin. When rotated in a counterclockwise (CCW) manner, the flagella provide a powerful forward thrust. However, if the rotary direction is flipped to a clockwise (CW) direction, a polymorphic change is induced that causes a right-angled bend in the filament to propagate along the flagella. This compels the bundle of flagella to separate, causing a period of rotational movement due to the low impact of inertia at microbial scales. This process is called a tumble and allows bacteria to change their direction of movement (Macnab and Aizawa, 1984; Armitage and Schmitt, 1997; Berg, 2003; Stock et al., 2012; Wang et al., 2012). The “run and tumble” mechanism produces a movement called a “biased random walk,” with the rate of tumbles being random but biased by the chemotaxis system (Sourjik and Wingreen, 2012).

In contrast, marine bacteria such as *Vibrio alginolyticus* tend to reverse their swimming direction instead of tumbling, by stably rotating their polar or subpolar flagella in the opposite direction and dragging the cell body. This “run and reverse” mechanism can also produce a “biased random walk” by controlling the frequency of reversal movements, although it is reliant on Brownian motion to reorient the cell body (Mitchell et al., 1996). *Vibrio* spp. (including *V. alginolyticus*) were recently discovered to also “flick” the cell body after a reversal, caused by buckling of the hook adapter, and resulting in a tumble-like random reorientation of the cell (Xie et al., 2011; Son et al., 2013; Taktikos et al., 2013). Indeed, up to 60% of marine bacteria may actually be performing “run, reverse, and flick” motility (Son et al., 2013).

The Complex Flagella of *Sinorhizobium meliloti* and *Agrobacterium fabacearum*

S. meliloti and *A. fabacearum* are both well-studied Gram-negative swimming bacteria. Although their flagella are built in a similar fashion to those in *E. coli* and in similar numbers, there are several important differences (see **Figure 1B**). The main difference is the shape of the filament; *S. meliloti* and *A. fabacearum*'s filaments are formed by right-handed helices and as such forward thrust is produced by CW rotation (Gotz et al., 1982; Armitage and Schmitt, 1997; Sourjik and Schmitt, 1998). They also have increased rigidity, likely due to the use of several distinct flagellin proteins. Half of the flagella mass consists of the FlaA protein which forms heterodimers with two or three other proteins: FlaB, FlaD and, in *S. meliloti*, FlaC (Scharf et al., 2001). The rigid *S. meliloti* and *A. fabacearum* flagella may be adapted for use in the relatively viscous medium of soil, compared to the native habitat of *E. coli*, the human gut (Armitage and Schmitt, 1997; Attmannspacher et al., 2005). In addition, they are unable to switch the rotary direction of the motor. Instead, they modulate the speed of rotation through an extra stator protein, MotC, that binds MotB in the periplasm and may, thus, regulate the speed of the motor. A further

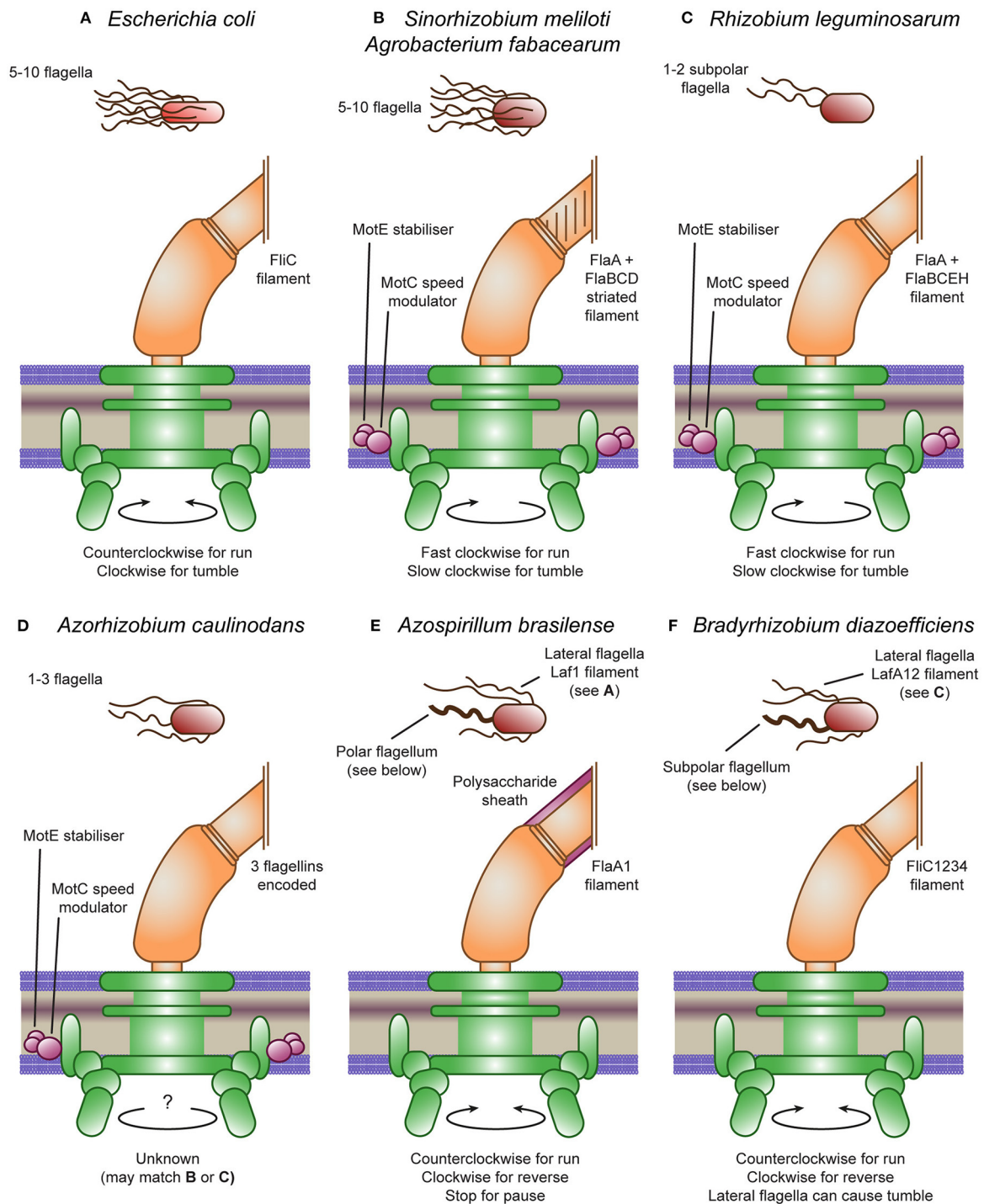


FIGURE 1 | *Escherichia coli* and rhizobial motility models. **(A)** The *E. coli* peritrichous flagella provides thrust through counterclockwise rotation and tumbles through clockwise rotation causing the flagella bundle to dissociate. **(B)** The *Sinorhizobium meliloti* and *Agrobacterium fabacearum* peritrichous flagella provide thrust through clockwise rotation and tumbles through speed modulation causing the flagella bundle to dissociate. This speed modulation is controlled by MotC, stabilized by MotE. The reversed direction is due to the heterogeneous filament proteins producing a right-handed striated filament. **(C)** The *Rhizobium leguminosarum* subpolar flagella also provide thrust through clockwise rotation and tumbles through speed modulation, although they do not form a flagella bundle. The *R. leguminosarum* flagella also have heterogeneous filaments, although they are not visibly striated. **(D)** The *Azorhizobium caulinodans* flagella are not well-studied but may match either **(B)** or **(C)**, having the MotC and MotE accessory proteins. **(E)** *Azospirillum brasilense* produces two flagella types: a polar flagellum covered by a polysaccharide sheath that provides thrust through counterclockwise rotation, reverses through clockwise rotation and can pause by stopping the motor; and lateral flagella matching **(A)**. **(F)** *Bradyrhizobium diazoefficiens* produces two flagella types: a subpolar flagellum that provides thrust through counterclockwise rotation and reverses through clockwise rotation; and lateral flagella matching **(C)** that can cause tumbles.

additional protein, MotE, stabilizes MotC and may target the protein to the flagellar motor (Eggenhofer et al., 2004). The product of another nearby gene was originally designated as the novel motor protein MotD; however, mutational analysis revealed its function to be that of FliK, the flagellar hook length regulator that controls the switch from secretion of hook-type substrates to filament-type substrates (Eggenhofer et al., 2006).

Despite being unable to cause a tumble in the same way as *E. coli* due to their unidirectional flagella, *S. meliloti* and *A. fabacearum* can still tumble. The bacteria achieve this by asynchronously modulating the rotational speed of their flagella, causing their bundle of flagella to disassociate, causing a tumble (Armitage and Schmitt, 1997; Attmannspacher et al., 2005). The ability to modulate the speed of their flagella also enables the bacteria to increase cell velocity in addition to reducing tumbling in response to high attractant concentrations (Meier et al., 2007).

The Smooth Flagella of *Rhizobium leguminosarum* and *Azorhizobium caulinodans*

Motility in *R. leguminosarum* and *A. caulinodans* appears to be closely related to that in *S. meliloti*, containing all the accessory genes (see **Figures 1C,D**). *R. leguminosarum* appear to only have 1-2 subpolar flagella with a smooth surface, lacking the helical perturbations found in *S. meliloti* (Tambalo et al., 2010a). *A. caulinodans* also have 1-3 smooth flagella, although they are arranged around the cell in a peritrichous arrangement (Liu et al., 2018a). The protein structure of the *R. leguminosarum* filament is closely related to *S. meliloti*, again consisting of heterologous pairs of a main flagellin (FlaA) and multiple secondary flagellin (FlaB, FlaC, FlaE, and FlaH) (Scharf et al., 2001; Tambalo et al., 2010a). Indeed, this bacterium is also unable to change the direction of rotation, so directional changes are driven by modulating the rotational speed of a single or pair of flagella, although the mechanism is currently unknown (Miller et al., 2007). However, *R. leguminosarum* still appears to tumble approximately every 3 s under homogeneous conditions, while it swims at approximately 38 $\mu\text{m/s}$ (Miller et al., 2007). The protein structure of the *A. caulinodans* filament is unknown, but it does encode three flagellin copies. In addition, although the rotation direction of the flagella of *A. caulinodans* is unknown, since it encodes both *motC* and *motE*, it likely also modulates the speed of rotation (Lee et al., 2008).

The Composite Flagellar Systems of *Azospirillum brasilense* and *Bradyrhizobium diazoefficiens*

Both *A. brasilense* and *B. diazoefficiens* encode two flagella systems, one producing a single polar or subpolar flagellum and the other producing multiple lateral flagella (see **Figures 1E,F**) (Zhulin and Armitage, 1993; Kaneko et al., 2002). The flagellins forming the lateral flagella are encoded by *laf1* in *A. brasilense* and, *lafA1* and *lafA2* in *B. diazoefficiens* and are all 300–400 amino acids long, similar in size to other rhizobia (Moens et al., 1995a,b; Kanbe et al., 2007). In contrast, the flagellins forming

the polar flagella are encoded by *fla1* in *A. brasilense* and, *fliC1*, *fliC2*, *fliC3*, and *fliC4* in *B. diazoefficiens* and are all 600–800 amino acids long, producing thicker filaments with bidirectional rotation (Zhulin and Armitage, 1993; Quelas et al., 2016). In addition, the lateral flagella of *B. diazoefficiens* were found to be more closely related to the flagella of other rhizobia than the divergent polar flagellum (Garrido-Sanz et al., 2019). Indeed, the *B. diazoefficiens* lateral flagella can only rotate in a single direction and their cluster also encodes *motC* and *motE* (Kanbe et al., 2007). The rotation direction of the *A. brasilense* lateral flagella is unknown but the cluster does not encode *motC* and *motE*. A further difference is that the polar flagellum of *A. brasilense* is covered with a polysaccharide sheath not found in other rhizobia (Moens et al., 1995b; Burygin et al., 2007; Belyakov et al., 2012). Glycosylation of flagella has been linked to avoiding plant immunity in *Pseudomonas syringae* (Takeuchi et al., 2003; Taguchi et al., 2009; Iwashkiw et al., 2013). In contrast, other rhizobial flagellin are sufficiently divergent from pathogenic flagellins to avoid a plant response (Felix et al., 1999). Perhaps the polar flagellum of *A. brasilense* requires glycosylation to prevent plant detection, in which case, the subpolar flagellum of *B. diazoefficiens* may be similarly glycosylated. Two flagellin modification genes, *flmA* and *flmB*, were found to be essential for the formation of the *A. brasilense* polar flagellum (Rossi et al., 2016). In addition to having a similar composite system, the physical properties of the *A. brasilense* and *B. diazoefficiens* lateral flagella and separately their polar flagella were found to be nearly identical and were classified into the same groups (Fujii et al., 2008). In contrast, the *S. meliloti* flagella were divergent from these groups, potentially due to their complex, striated structure (Fujii et al., 2008).

The swarming motility of both *A. brasilense* and *B. diazoefficiens* are driven mainly by the lateral flagella, with *A. brasilense* only producing lateral flagella on solid or semi-solid surfaces (Zhulin and Armitage, 1993; Covelli et al., 2013). The swimming motility of *A. brasilense* is mixed, following mainly “run and reverse” and “run, reverse, and flick” motility with occasional pauses and reduced swimming speed in response to attractants (chemokinesis) driven mainly by the polar flagellum (Zhulin and Armitage, 1993; Mukherjee et al., 2019). The swimming motility of *B. diazoefficiens* is similarly mixed, following 50% “run and reverse,” 30% “run, reverse, and flick” and, interestingly, 20% “run and tumble” (Quelas et al., 2016). The reversals and flicks were driven by the polar flagellum, whereas the tumbles were produced by the lateral flagella. The composite flagellar systems of *B. diazoefficiens* thus produce a composite motility performance.

Rhizobial Pili Systems

In addition to flagella, *S. meliloti*, *A. fabacearum*, *R. leguminosarum*, *A. brasilense* and *B. diazoefficiens* encode type IV pili on their chromosomes, with *S. meliloti* and *B. diazoefficiens* encoding additional, truncated clusters (Krehenbrink and Downie, 2008; Wibberg et al., 2011; Wisniewski-Dye et al., 2011; Zatakia et al., 2014; Mongiardini et al., 2016). The main cluster of *B. diazoefficiens* is split with the *tadE*, *tadF*, and *tadG* genes located almost 3 Mb away from the remaining genes

(Mongiardini et al., 2016). *A. caulinodans* does not encode a pili system (Lee et al., 2008). Each species' main cluster displays high homology to the *tad* systems of *Aggregatibacter actinomycetemcomitans* and *Caulobacter crescentus* (Tomich et al., 2007; Clock et al., 2008; Imam et al., 2011). Beyond bioinformatic characterization, very little research has been conducted on rhizobial pili systems. *A. brasilense* was found to form polar pili bundles that are required for biofilm formation (Shelud'ko and Katsy, 2001; Wisniewski-Dye et al., 2011). Interestingly, deletion of *pilA1*, the integral pilin subunit of the chromosomal cluster of *S. meliloti*, was found to reduce competitive nodulation of *Medicago sativa* plants (Zatakia et al., 2014). In addition, the TadG protein of *B. diazoefficiens* has some sequence similarity to *Bradyrhizobium* lectin BJ38, which is important for soybean root adhesion (Loh et al., 1993; Ho et al., 1994; Mongiardini et al., 2016). This indicates that there is a role for pili in legume symbiosis, perhaps during root colonization.

The *Escherichia coli* Chemotaxis Model

Chemotaxis involves regulating motility through variations in the concentration of metabolically-relevant chemicals, being either attractants (compounds which benefit the bacteria) or repellents (those compounds with negative effects). Bacterial size is insufficient to spatially sense concentration gradients at swimming speeds so, instead, concentrations are compared temporally (Porter et al., 2011). Bacteria bias their movements toward high concentrations of attractants by reducing their tumbles, and away either from low concentrations of attractants or high concentrations of repellents by increasing their tumbles.

The core of the chemotaxis response in *E. coli* is the phosphorylation of the response regulator CheY by the histidine kinase CheA in response to negative signal transduction from the chemoreceptor proteins. The chemoreceptors tend to sense in the periplasm and form a coiled coil of about 30–40 heptads (7 amino acid repeats) in the cytoplasm (forming the categories 34H, 36H, 38H, and 40H) and bind CheA at the base (Figure 2A) (Wuichet and Zhulin, 2010). The length of the coiled coil determines the association between chemoreceptors, with those of the same length forming hexagonal arrays (Jones and Armitage, 2015). The binding of CheA to the chemoreceptors within these hexagonal arrays is stabilized by CheW. Phosphorylated CheY binds the motor protein FliM, reducing rotary speed and inducing a tumble (Sourjik and Schmitt, 1998). In addition, the chemotaxis proteins CheR and phosphorylated CheB methylate and demethylate chemoreceptors, reducing and increasing their signal sensitivity, respectively (Rice and Dahlquist, 1991; Armitage and Schmitt, 1997; Porter et al., 2011). *E. coli* encodes an additional chemotaxis protein, CheZ, which interacts with CheY to remove phosphorylation, enabling the cell to quickly adapt to new conditions.

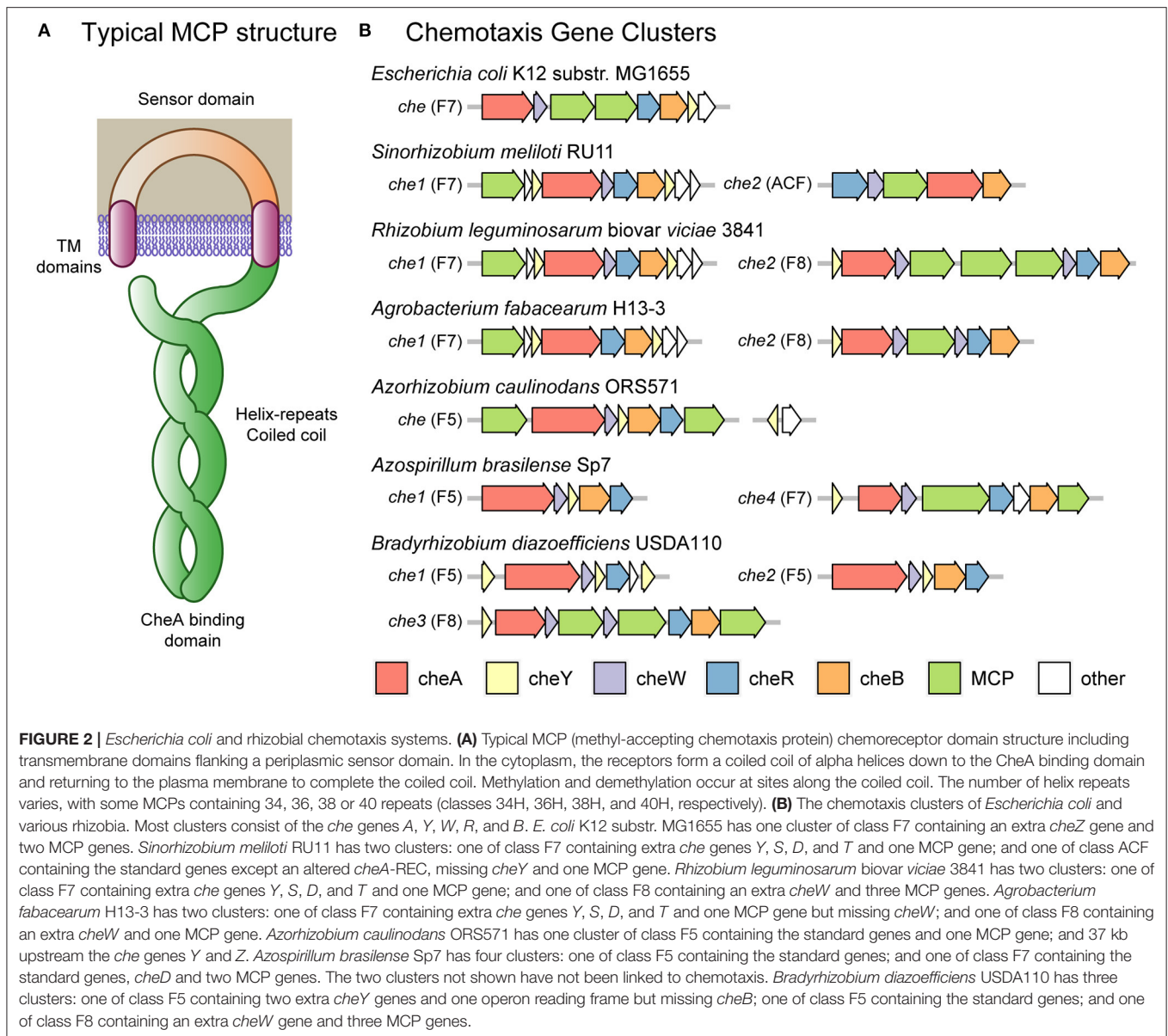
Chemotaxis Systems in Symbiotic Bacteria

In contrast to *E. coli*, most of the rhizobia encode multiple chemotaxis systems. The rhizobia also encode a diversity of chemoreceptors divergent from the 5 chemoreceptors encoded by *E. coli* K12, with the motility model strain *S. meliloti* RU11

encoding 8 chemoreceptors, *A. fabacearum* H13-3 encoding 22, *R. leguminosarum* biovar *viciae* 3841 encoding 26, *A. caulinodans* ORS571 encoding 43, *A. brasilense* Sp7 encoding 41, and *B. diazoefficiens* USDA110 encoding 36 (Rebbapragada et al., 1997; Yost et al., 2004; Jiang et al., 2016; Scharf et al., 2016; Zatakia et al., 2017). However, the main chemotactic systems of *S. meliloti*, *A. fabacearum*, and *R. leguminosarum* have a similar core system to that of *E. coli*, belonging to class F7 and being associated with chemoreceptors of type 36H (Sourjik and Schmitt, 1998; Miller et al., 2007; Tambalo et al., 2010a,b; Wibberg et al., 2011) (see Figures 2A,B). These species do not encode CheZ, instead they encode two copies of CheY, with CheY2 propagating the signal and CheY1 acting as a phosphate sink, increasing the rate at which CheY2 returns to its unphosphorylated state (Sourjik and Schmitt, 1998). A further rhizobial protein, CheS, forms a dimer that binds CheA and CheY1, increasing phosphate transfer and thus CheY2 dephosphorylation (Dogra et al., 2012; Arapov et al., 2020). The rhizobia also encode two uncharacterized chemotaxis proteins: CheD and CheT. In the case of CheD, this protein has homology to *Bacillus subtilis* CheD protein which was found to deamidate chemoreceptors, a role served by CheB in *E. coli* (Kristich and Ordal, 2002; Rao et al., 2008). In *S. meliloti*, *cheT* mutants have a chemotaxis defect, indicating that it is likely to have some relevant, although currently unknown, chemotactic role (Arapov et al., 2020).

A. caulinodans, in contrast, only encodes a single chemotaxis system belonging to class F5 and being associated with chemoreceptors of type 38H. This bacterium encodes a cluster with *cheY*, *cheA*, *cheR*, *cheB*, *cheW* and two chemoreceptors (Jiang et al., 2016). In addition, it has a separate cluster located 37 kb upstream and encoding a second copy of the *cheY* gene and a *cheZ* gene. CheZ appears to be a functional protein with a conserved phosphatase motif critical for chemotactic activity (Liu et al., 2018b). Both of the CheY proteins appear to be active, with the main operon *cheY1* mutant displaying a reduced tumble rate, causing a 40% reduction in the chemotaxis swimming halo on semi-solid media, and a *cheY2* mutant displaying an increased tumble rate with a 90% reduction in the chemotaxis swimming halo (Liu et al., 2020).

In addition to the main systems described above, both *A. fabacearum* and *R. leguminosarum* encode accessory chemotaxis systems. These systems are F8-class chromosomal clusters encoding *cheY*, *cheA*, *cheR*, *cheB*, two *cheW* genes with one chemoreceptor (*mcpB*) in *A. fabacearum* and three in *R. leguminosarum*. These three chemoreceptors of *R. leguminosarum* (*mcrA*, *mcrB*, and *mcrC*) are of type 34H, indicating that it is an independent sensory system to the 36H chemoreceptors of Che1 (see Figure 2A). The class of the chemoreceptor encoded by *A. fabacearum* has not been determined, although this protein is likely to match *mcrA*, *mcrB*, and *mcrC* of *R. leguminosarum* due to the chemotaxis cluster arrangements. The *R. leguminosarum* Che2 cluster has a minor role in chemotaxis in free-living conditions (Miller et al., 2007). However, a recent insertion sequencing experiment in *R. leguminosarum* bv. *viciae* 3841 found that insertions in any of the *che2* cluster genes were over-represented in nodule bacteria samples, suggesting a role in symbiosis (Wheatley et al., 2020).



S. meliloti also encodes an accessory chemotaxis system located on its pSymA plasmid, which belongs to the alternative cellular function (ACF) class and encodes only *cheR*, *cheB*, *cheW*, a chemoreceptor (*mcpS*) and a *cheA* gene fused with a response-regulator (REC) domain (Wuichet and Zhulin, 2010; Scharf et al., 2016). This cluster is associated with a chemoreceptor of type 40H, *mcpS*, which is divergent from the other *S. meliloti* chemoreceptors of type 36H (see **Figure 2A**). The *mcpS* gene was not expressed in free-living cells, indicating that this chemotaxis operon is not active under those conditions (Meier and Scharf, 2009; Scharf et al., 2016). In addition, the absence of a *cheY* gene indicates that the system does not control flagella; instead, it is likely that the modified CheA-REC protein regulates downstream effectors.

Chemotaxis Systems Controlling “Run and Reverse” Motility

In addition to multiple flagellar systems, *A. brasilense* has four chemotaxis clusters and *B. diazoefficiens* has three (see **Figure 2B**). Three of the four chemotaxis systems of *A. brasilense* (*che1*, *che2*, and *che4*) include the core *cheY*, *cheA*, *cheR*, *cheB*, and *cheW* genes. The *che1* system of *A. brasilense* is an F5-class cluster associated with chemoreceptors of type 38H (of which there are 33 in the genome), harboring the core genes above, including a *cheA* gene fused with a response-regulator (REC) domain (Wuichet and Zhulin, 2010). The *che2* system is an F9-class cluster containing the core genes mentioned above, including a fragmented *cheA* in addition to another *cheY* and a *cheC* phosphatase gene. The *che3* system is an ACF-class

cluster only encoding a fragmented *cheA*, *cheB*, *cheW* genes, one chemoreceptor and a putative histidine-kinase response-regulator pair. Finally, the *che4* system is an F7-class cluster associated with chemoreceptors of type 36H (of which there are 8 in the genome), encoding the core genes and a *cheD* gene. *A. brasilense* has been observed to react with reverses and pauses and to modulate speed in response to chemical gradients (Zhulin and Armitage, 1993; Mukherjee et al., 2019). Numerous studies have revealed that the Che4 system modulates reversals by changing the rotation direction of the polar flagellum, while the Che1 system modulates swimming speed (Zhulin and Armitage, 1993; Bible et al., 2012; Mukherjee et al., 2016; Ganusova et al., 2021). In addition, the Che4 system, in concert with two orphan CheY proteins (CheY6 and CheY7), also modulates the pausing behavior, aerotaxis and the induction of swarming motility (Mukherjee et al., 2019; Ganusova et al., 2021). The Che2 and Che3 systems do not appear to be involved in chemotaxis. Regarding the three *B. diazoefficiens* chemotaxis clusters (*che1*, *che2*, and *che3*), they include the core *cheY*, *cheA*, *cheR*, *cheB*, and *cheW* genes, except the *che1* F5-class system, which does not contain a *cheB* gene but an additional two *cheY* genes. The *che2* system is also an F5-class cluster including the core genes. The *che3* system is an F8-class cluster associated with chemoreceptors of type 34H and encodes an additional *cheW* gene with three chemoreceptors (Kaneko et al., 2002; Wuichet and Zhulin, 2010). The involvement of these systems on chemotaxis is currently unknown, although it was found that only the subpolar flagellum of *B. diazoefficiens* responds chemotactically to glutamate and succinate (Quelas et al., 2016). This indicates that the primary chemotactic response is via the subpolar flagellum, similar to *A. brasilense*.

Regulation of Motility and Chemotaxis

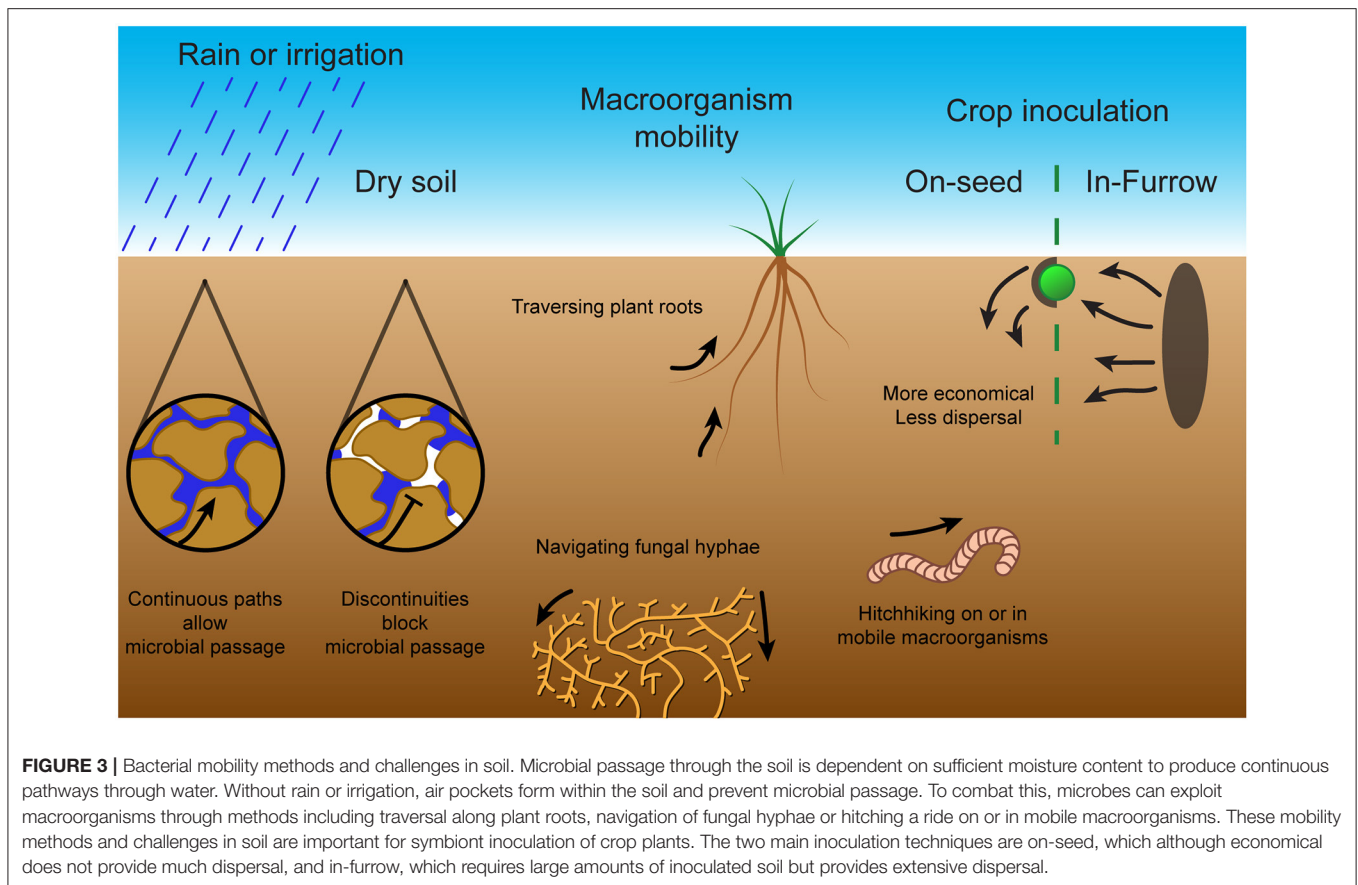
S. meliloti, *A. fabacearum*, and *R. leguminosarum* all have the same transcriptional activation system for flagellar and chemotaxis genes. Regulation of flagella in bacteria typically follows a hierarchical class system: regulatory class I genes control the transcription of class II genes required to initiate flagellar assembly and which, in turn, regulate class III genes to complete assembly and perform chemotaxis (McCarter, 2006). This class system ensures that each gene is active only when its product is useful. In the above rhizobia, the class I heterodimer VisNR is induced by some unknown effector to activate both promoters of *rem* and negatively regulate *visN* (Rotter et al., 2006). In turn, Rem activates the transcription of class IIA downstream regulators *fliM* (encoding a motor protein) and *orf38* (encoding a potential basal body protein) in addition to flagellar assembly and motor genes (class IIB) (Tambalo et al., 2010b). Presumably activated through some signal of basal body completion, FliM and Orf38 then activate flagellin and chemotaxis genes (class III), to produce functional flagella with chemotactic ability. Mutants in *visN*, *visR*, and *rem* are nonmotile and non-flagellated, indicating that they form the master regulators for flagella in *S. meliloti* and *R. leguminosarum* (Sourjik et al., 2000; Rotter et al., 2006; Tambalo et al., 2010b). Although all of these genes are encoded by *A. fabacearum*, their function has not yet been confirmed. In *R. leguminosarum*, VisNR and Rem are expressed

throughout exponential growth phase, but their expression drops sharply during stationary phase; although the cells are still motile, likely retaining their flagella due to the stability of the filament (McCarter, 2006; Tambalo et al., 2010b; Zhuang and Lo, 2020; Nedeljkovic et al., 2021). The only motility and chemotaxis genes in *R. leguminosarum* that are independent of VisNR are scattered chemoreceptors and, notably, the *che2* cluster (Scharf et al., 2016). This possibly allows the bacteria to target the expression of *che2* genes independently of that of motility genes and indicates that the role of the Che2 cluster may not be linked to flagella.

Neither *A. caulinodans* nor *B. diazoefficiens* encode *visNR*, although both encode homologs of *rem* (Lee et al., 2008; Mongiardini et al., 2017). The *B. diazoefficiens* Rem-homolog LafR was recently found to control transcriptional regulation of its lateral flagella but not its polar flagellum. The orphan response regulator did not require phosphorylation for its effect although it was activated by arabinose and oxidative stress while being repressed by mannitol, oxygen limitation, and iron deficiency (Mongiardini et al., 2017). LafR was found to activate genes across multiple operons, including *flbT*, the gene product of which translationally induces the LafA1 and LafA2 lateral flagellins (Mongiardini et al., 2017). Although the role of *rem* in *A. caulinodans* is unknown, some recent work has indicated that the flagella and chemotaxis genes have increased transcription in the presence of the amino acids histidine, arginine and aspartate (Liu et al., 2019). *A. caulinodans* displays chemotaxis toward these amino acids, that are sensed by the chemoreceptor TlpH, although the *tlpH* mutant did not remove the transcriptional effect (Liu et al., 2019). The mechanistic link between the amino acids and flagellar transcriptional regulation remains unknown. In contrast to the other rhizobia, *A. brasilense* does not encode *visNR* or *rem*. Instead, it appears that the nitrogen regulator NtrA controls flagellar gene transcription via a σ^{54} box. Indeed, a *ntrA* mutant was found to be non-flagellated (Moens et al., 1995a).

Importance of Chemotaxis and Motility Systems for Rhizobia as Free-Living Bacteria in Soil Conditions

Bacterial movement through soil depends on continuous water pathways with their inability to traverse air pockets (Griffin and Quail, 1968) (see **Figure 3**). Overall, with insufficient soil water content, microbial mobility is limited, whereas overloaded soil has greatly reduced oxygen concentrations (Tecon and Or, 2017). The water content in the soil has been described by different measures including matric potential, moisture content and percentage field capacity which all increase with increasing water content. For example, the swarming motility of various *Pseudomonas* species was found to be restricted to high matric potential agar with very high moisture content, showing a fast transition to non-motility upon drying (Dechesne and Smets, 2012). The soil bacterium *Pseudomonas aeruginosa* displayed reduced motility with decreases in soil water content (increased matric suction), to the point of non-motility (Griffin and Quail, 1968). *Rhizobium trifolii* motility also slowed with increasing water tension and was halted by discontinuity; conversely, the mean radius of the bacterial motility area increased with



increased water content (Hamdi, 1971). The bean symbiont *Rhizobium leguminosarum* bv. *phaseoli* displayed a reduction in travel distance in soil with reduced moisture content such that it resembled a non-flagellated, non-motile mutant strain (Issa et al., 1993). The soil bacterium *Pseudomonas fluorescens* had reduced horizontal and vertical migration with decreased water irrigation flow rate (Singh et al., 2002). In addition, soil bacteria *A. brasilense* and *P. fluorescens* displayed increased migration in soil with large continuous pores (sand) over soil with narrow water channels (clay or loamy soils) (Bashan, 1986; Singh et al., 2002). The reduction in microbial dispersal with reduced soil water content implies that the usual state of soil bacteria is sessility imposed by the ubiquitous unsaturated state of soils (Tecon and Or, 2017). Thus, motility is limited in importance to temporary flooded states of soil formed immediately after rainfall or irrigation.

An effective rhizobial inoculant must survive in the competitive environment of the soil (Atieno and Lesueur, 2018). Water content was again found to be a key determinant for survival in the soil. Soil with higher moisture content and soil types with high field capacity (total water capacity of the soil) were found to support larger populations of *B. japonicum* and *R. leguminosarum* (Mahler and Wollum, 1981). *P. fluorescens* also had increased survival in soil with increased moisture content (van Elsas et al., 1991). At microbial scale, however, the

soil is dynamic and fragmented with changes in soil hydration and pore-spaces dramatically influencing their dispersion and fluctuating nutrient gradients attracting them down fleeting paths. This produces a heterogeneity in microsites that is averaged out by macro studies at the cm^3 scale (Tecon and Or, 2017). For instance, motile *E. coli* was found to accumulate within funnel-shaped microsites; thus, porous wet soil could cause heterogenous arrangements of bacteria simply by their shapes (Galajda et al., 2007). Theoretical models have indicated that in heterogeneous environments, a more motile bacteria can outgrow a metabolically superior species (Lauffenburger et al., 1982). Therefore, an elite inoculant would need to have powerful but adaptable motility and chemotaxis systems.

There are additional complications to chemotaxis control of motility in the soil. The control exerted by chemotaxis systems on bacterial motility does provide an improvement over random cell motility, with chemotactic strains having 10 times faster predicted soil dispersal rates (Ebrahimi and Or, 2014). Indeed, *P. fluorescens* was found to migrate further in soil irrigated by chemoattractant-rich fungal exudates when compared to sterile water (Singh et al., 2002). The soil bacteria *A. brasilense* and *P. fluorescens* migrate toward roots guided by chemotaxis at increased rates with increased water content up to the water capacity of the soil (Bashan, 1986). In contrast, the symbiont *B. diazoefficiens* was found to swarm quickly through wet

soil with no supplemented chemoattractants (Covelli et al., 2013). Chemotactic detection of nutrients in soil is limited by fragmentation of water pockets due to drainage, plant uptake and evaporation, factors limiting nutrient diffusion and gradient establishment. Thus, reduction in soil matric potential—reducing moisture content—exponentially decreases chemotactic movement (Ebrahimi and Or, 2014).

Since active movement through dry soil is severely limited, other passive forms of movement dominate (see **Figure 3**). These include environmental forces, such as water percolation or wind dispersal of soil particles on the surface, in addition to “hitchhiking” on larger organisms such as earthworms, nematodes, protozoa, soil fungal networks or plant roots (Yang and van Elsas, 2018; King and Bell, 2021). This “hitchhiking” occurs through either adhesion to macroorganism surfaces, or through consumption and survival in the gut with subsequent release into the soil by expulsion or death of the macroorganism. For instance, *S. meliloti* migration through sterile soil was improved by the presence of *Caenorhabditis elegans* nematode worms, and migration of *B. japonicum* and *Pseudomonas putida* was improved by the presence of burrowing earthworms (Madsen and Alexander, 1982; Horiuchi et al., 2005). On the other hand, excessive adhesion may reduce dispersal, as *E. coli*, *Rhodococcus erythropolis* and *P. putida* species were found to be retained more in more adhesive soils with reduced vertical dispersal by water percolation (Jimenez-Sanchez et al., 2015; Sepehrnia et al., 2019). In addition, migration along hyphal networks or across plant roots enables mobility despite discontinuities in the surrounding soil pores (Yang and van Elsas, 2018). *Rhizobium leguminosarum* bv. *trifolii* was found to migrate further in presence of clover plant roots and *P. fluorescens* displayed greatly increased depth and magnitude of soil colonization in the presence of wheat plant roots (van Elsas et al., 1991; Worrall and Roughley, 1991). The range and depth of *A. brasilense* soil colonization was also increased by the presence of both wheat and weed roots (Bashan and Levanony, 1987). Thus, the importance of bacterial motility and chemotaxis in the soil is reliant on favorable environmental factors.

Importance of Chemotaxis and Motility Systems for Plant Colonization and in Symbiosis

Motility and chemotaxis also appear to be crucial for competitive root surface colonization and for the establishment of a successful symbiosis (Catlow et al., 1990; Yost et al., 1998; Miller et al., 2007; Scharf et al., 2016). Indeed, most symbionts of eukaryotes have flagellar and chemotaxis capabilities (Raina et al., 2019). However, experiments were performed in well-watered pots that may not truly reflect the field environments in which these symbionts are applied as inoculants. There have been hints that macro-scale mechanisms, such as water percolation, are required to enable bacterial mobility and for effective colonization and nodulation. For example, in low-moisture soil, the symbiont *R. trifolii* inoculated on the seed displayed delayed infection thread formation and nodulation of *Trifolium subterraneum* plants that was partially recovered by watering (Worrall and Roughley,

1976). Vertical transportation of the symbiont *B. japonicum* and the soil bacterium *P. putida* in soil was found to be limited even in the presence of developing soybean and bean plant roots with the absence of percolating water or burrowing earthworms (Madsen and Alexander, 1982). The colonization and nodulation competitiveness of *R. leguminosarum* bv. *viciae* on lentil plants was also found to decrease with depth in non-sterile soil but to partially recover with increased soil moisture content (Karmakar and Chandra, 2012). Finally, various motile *B. diazoefficiens* strains (LP3001 and LP3008) out-competed their non-motile derivatives at soybean plant nodulation under flooded conditions but not when merely at field capacity (Althabegoiti et al., 2011).

A bacterium can achieve a growth advantage by arriving and establishing itself at the root surface before competing bacteria. In addition, flagella-based swarming motility enables movement along the surface of the root for more effective colonization (Simons et al., 1996; Gao et al., 2016). Indeed, motility and chemotaxis mutants of the soil bacterium *B. subtilis* display greatly reduced colonization on *Arabidopsis thaliana* roots after 4 h and on tomato roots after 2 weeks (Allard-massicotte et al., 2016; Gao et al., 2016). In addition, a *P. fluorescens* motile strain out-competed a non-motile strain in root colonization and attachment in sterile soil, whereas transfer of *A. brasilense* through sand from wheat to soybean roots was only observed with the motile strain (Bashan and Holguin, 1994; Turnbull et al., 2001). In contrast, *P. fluorescens* 2-79 RN10 was also found to traverse further along wheat plant roots when inoculated at the root apex rather than the seed, indicating that the bacteria moved with root growth (Parke et al., 1986). Thus, while motility and chemotaxis are integral systems for a competitively colonizing inoculant, they are not the sole mechanism for soil dispersal.

Dispersal of Symbionts as Crop Inoculants in Field Conditions

Farming techniques alter soil microbial diversity and abundance by changing soil composition through crop cultivation or fertilizer application or by disrupting aggregate structures through tillage (Tecon and Or, 2017). Thus, it is important that any inoculants used are tested in real-world field use and are applied in an efficient and effective manner. Current inoculants tend to be applied either on-seed as a coating or in-furrow within the soil. Seed inoculation is more economical, so it has been used more extensively, although some studies indicate that in-furrow inoculation provides improved dispersal of the inoculant (Deaker et al., 2012; Iturralde et al., 2019). For instance, in field soil multiple symbiotic *B. japonicum* strains inoculated on-seed were found to have reduced nodule occupancy compared to in-furrow inoculation, particularly at depth and on lateral roots (McDermott and Graham, 1989; Wadisirisuk et al., 1989; Bogino et al., 2011). In addition, *B. japonicum* LP3001 established in vermiculite soil greatly out-competed (in soybean root nodulation) the equally competitive *B. japonicum* LP3004 inoculated on the seed (Lopez-Garcia et al., 2002). Interestingly, the seed-inoculated *Bradyrhizobium*

had 50% reduced colonization in the mid root and apical root sections, such that slow vertical displacement of the seed-inoculated strain was identified as a major factor in the relative competitiveness of the strains (Lopez-Garcia et al., 2002). In further experiments with a hyper-motile strain, it was found that in-furrow inoculation of *B. japonicum* LP3008 could also considerably improve soybean root nodulation competitiveness compared to seed inoculation in field soil conditions (Lopez-Garcia et al., 2009). In addition, although on-seed inoculation of *R. leguminosarum* bv. *viciae* on faba bean and *Bradyrhizobium lupini* on lupin produced more and larger nodules than in-furrow inoculation at more than half of the field sites tested, potentially these nodules were larger because they were taproot nodules initiated earlier (Denton et al., 2017). Denton et al. (2017) also found that, in general, rhizobial inoculation improved taproot nodulation, whereas only improved lateral root nodulation in sites with reduced native rhizobia. Thus, the effectiveness of these two application techniques may depend on both the field conditions and the native rhizobial population. To improve the competitiveness of inoculants, other strategies include in-field dispersal in porous bags filled with inoculated soil which enable multiple establishment attempts or by leveraging interactions with motile organisms or fungal hyphal networks to promote dispersal (King and Bell, 2021). However, selecting for excess dispersal could actually be counterproductive by diluting inoculants away from the intended targets.

DISCUSSION

Regardless of the complexity and importance of motility and chemotaxis in the soil environment, there is a clear selection for chemotaxis genes in wildtype rhizobia. Analysis of 264 completed bacterial genomes gave an average chemoreceptor gene count of 14, which was found to be increased in species with high metabolic diversity, low stability of habitat and those with interactions with other living species (Lacal et al., 2010). The category with the highest number of chemoreceptors included soil-dwelling organisms such as *Azospirillum lipoferum* or *Bradyrhizobium* sp. strain BTAi1 encoding 63 and 60 predicted chemoreceptors, respectively, and several plant-interacting bacteria, including *A. caulinodans* ORS571 encoding 43 genes, *R. leguminosarum* bv. *viciae* 3841 encoding 26 genes and *B. diazoefficiens* USDA110 encoding 36 genes (Scharf et al., 2016). In contrast, *E. coli*, a human gut microbe, has only 5 chemoreceptor genes. A mutant of *Azorhizobium caulinodans* ORS571 with the chemotaxis cluster deleted was defective in *Sesbania rostrata* root surface colonization and competitive nodulation (Liu et al., 2018a). In *R. leguminosarum* biovar *viciae* VF39SM, deletion of the chemotaxis receptors *mcpB* or *mcpC* resulted in a significant reduction of nodulation competitiveness on pea plants, although the ability to nodulate was not affected (Yost et al., 1998). Importantly, the VF39SM *mcpC* mutant does not have a competitiveness disadvantage in symbiosis with other plants, indicating that plant specificity is based on the perception of the different exudates each plant secretes and which accordingly drive a defined chemotaxis response (Scharf

et al., 2016). In the case of *S. meliloti* RU11, the chemoreceptors McpU and McpX have been found to target amino acids and quaternary ammonium compounds, respectively, being both present in the alfalfa host seed exudate (Webb et al., 2016, 2017a,b). Once inside the plant root, *S. meliloti* motility genes still appear to be expressed in bacteria in the infection thread channel but are completely down-regulated in bacteroids inside mature nodules (Yost, 1998). Interestingly, in other eukaryote-microbe symbioses, similar channels are generated to select for and guide their symbionts through generated gradients, via chemotaxis, toward the symbiosis space (e.g., the slime cavities of Hornwort or the ducts of the bobtail squid) (Raina et al., 2019). However, once inside the nodule, motility and chemotaxis are unnecessary for the now sessile bacteroids. This has been observed in *R. leguminosarum*, with down-regulation of chemoreceptors during bacteroid differentiation to undetectable amounts of expression in bacteroids (Yost et al., 2004; Karunakaran et al., 2009; Tambalo et al., 2010b). This regulation appears unrelated to the low oxygen conditions and organic acid availability inside the nodule (Yost et al., 2004; Tambalo et al., 2010b; Scharf et al., 2016). Thus, both motility and chemotaxis as dispersal strategies and competitive advantages are important aspects of symbiotic rhizobia.

As we have seen, chemotaxis and motility systems in rhizobia have a clear role both in free-living cells in soil and in symbiotic lifestyles, as indicated by their prevalence and the average number of receptor genes (Lacal et al., 2010; Scharf et al., 2016). However, bacterial movement through soil depends on continuous water pathways with their inability to traverse air pockets and, thus, the role of motility is limited to the temporary flooded states of soil formed immediately after rainfall or irrigation (Griffin and Quail, 1968). In addition, reduction in moisture content also decreases exponentially chemotactic movement by preventing the dispersal of chemoattractants and the formation of gradients (Ebrahimi and Or, 2014). Alternatively, environmental forces such as water percolation and the movement of larger organisms such as earthworms provide some degree of microbial dispersal, bypassing discontinuities in water channels (Yang and van Elsas, 2018; King and Bell, 2021). Another method used to bypass air pockets is traveling along eukaryotic pathways such as fungal hyphal networks and plant roots. Colonization of these networks can provide growth and settlement advantages, with chemotaxis and motility systems enabling the bacterium to arrive and establish before competing bacteria. Thus, although bacterial motility and chemotaxis are clearly important for soil competition, their importance is reliant on the favorable state of environmental factors.

The importance of motility and chemotaxis for elite inoculants—based on competitive and effective strains—is equally complex. Although motility and chemotaxis are integral systems for a competitively colonizing inoculant, their shortcomings mentioned above mean that they are not the sole mechanism for soil dispersal. It is thus important for any inoculant strain to be tested in field conditions and applied through efficient and effective methods. Of the two main techniques, it appears that on-seed inoculation is more economical but provides reduced dispersal opportunities, whereas in-furrow inoculation requires a larger quantity of

the inoculant but provides superior dispersal along crop root systems (Deaker et al., 2012; Tecon and Or, 2017). The effectiveness of both application techniques also seems to depend on both the field conditions and the native rhizobial population. Therefore, both motility and chemotaxis should be considered factors in the selection of elite inoculants, together with other dispersal strategies.

CONCLUSIONS

Improvements in symbiont inoculants depend on improvements in (1) understanding of motility and chemotaxis systems in rhizobia, (2) observations of motility and chemotaxis processes in the soil and (3) fast-throughput assays for individual inoculant selection. Firstly, more detailed knowledge of the chemotaxis and motility systems in rhizobia could determine the applicability of studies in other bacteria, such as *E. coli*, and enable mobility improvements to existing symbiont inoculants. For instance, improved knowledge of the many chemoreceptors with uncharacterized sensor domains could provide critical knowledge of the chemotactic signals that are critical for effective soil motility (Compton and Scharf, 2021). Additionally, assays that emulate field conditions, such as capillary and microfluidic assays, could be used to discover the decision-making logic of soil chemotaxis to allow targeted mimicry in engineered inoculants (Mao et al., 2003; Walsh et al., 2017; Lopez-Farfan et al., 2019). Secondly, improved observations of motility and chemotaxis processes in the soil would provide more detailed information about the heterogeneity of movement in the soil and rhizobial motility at the micro-scale. The micro-scale control of

motility in the soil has not previously been directly observed. Potentially, transparent pseudo-soils could enable visual tracking of mobility and dispersal from different inoculation techniques toward crop plants (Bhattacharjee and Datta, 2019; Ma et al., 2019). Finally, inoculant selection could be improved by the development of fast-throughput assays that could assess the dispersal effectiveness of an inoculant. For example, the work by Mendoza-Suarez et al. (2020) describes a fast-throughput assay to test the competitiveness and effectiveness of strains in a particular soil. Similar experiments could be devised to measure the field dispersal properties of candidate strains for inoculant formulations. In addition, they could enable more precise testing of other inoculation strategies, such as porous bag embedding or interactions with motile macroorganisms (King and Bell, 2021). These improvements could enable a faster and more effective generation work-flow for selecting or engineering symbiont inoculants by clarifying the importance of effective soil dispersal and dynamic chemotaxis and motility systems.

AUTHOR CONTRIBUTIONS

SA, CS-C, and PP conceived the project and wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Biotechnology and Biological Sciences Research Council [grant BB/M011224/1] and The Leverhulme Trust [grant RPG-2019-246].

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