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Characterization of a PilZ-Containing bacterial chemotaxis receptor in Azospirillum brasilense

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Abstract

Plant growth promoting bacteria that can be inoculated to important crops are possible sustainable alternatives to chemicals fertilizers *Azospirillum brasilense* is an alphaproteobacterium that is found in the rhizosphere and able to promote the growth of over 100 different plant species, including agriculturally important cereals and grains. Root surface colonization is a prerequisite for the bacterium-mediated plant growth promoting effect. Chemotaxis, the directed movement of motile *A. brasilense* in gradients is essential for plant root colonization and it depends on chemotaxis receptors that specifically recognize chemical cues. In *A. brasilense* the ability to move in gradients of oxygen, or aerotaxis, is a major motility response important for plant root surface colonization. Our lab has identified another chemoreceptor Aer that likely modulates aerotaxis. This receptor contains a PAS domain, two transmembrane domains, HAMP and MA, and a PilZ domain. PilZ domains are known to bind the secondary messenger c-di-GMP. Aer is hypothesized to mediate aerotaxis in *A. brasilense*. To determine the role of Aer in aerotaxis in *A. brasilense*, we constructed a deletion mutant and used functional complementation to characterize chemotaxis and aerotaxis. We found that Aer is essential for aerotaxis. Our findings suggest Aer and PilZ domains are critical for sensing elevated oxygen concentrations in a gradient which is important for supporting optimum metabolism of the microaerophilic *A. brasilense*.

Introduction

The rhizosphere is a chemical milieu. For plants to thrive in this complex environment, the must form mutualistic plant-microbe associations. Chemotaxis, the movement of motile cells in a direction corresponding to a chemical gradient, is a critical ability that enables motile soil bacterium to sense chemicals exuded by plants and locate them. Thus, chemotaxis a requirement for plant-root colonization (2).

Chemotaxis signals motile movement by activating internal molecular machinery. The physical behavioral response is guided movement towards or away from the chemical source via rotation of a flagella (3). This behavioral response is initiated by chemotaxis receptors. The signal is transduced to downstream chemotaxis cytoplasmic proteins that ultimately alter the direction of rotation of the flagellar motor. Soil bacteria are known to contain a plethora of chemotaxis receptors and multiple intracellular chemotaxis signaling systems (2).

Azospirillum are the most studied plant growth promoting bacteria and represents a common model for plant-microbe interactions (4). *Azospirillum brasilense* is a chemotactic alphaproteobacterium that is found in the rhizosphere and able to promote the growth of over 100 different plant species, including, but not limited to, agriculturally important cereals and grains (4). *A. brasilense*'s genome encodes four chemosensory operons, Che1, Che2, Che3, and Che4. Che1 regulates changes in swimming speed and also plays a role in chemotaxis. Che4 coordinates major signaling pathways for chemotaxis and wheat root colonization (5;6). Che2 and Che3 do not play roles in chemotaxis. To date only two chemoreceptors have been characterized: Tlp1 and AerC (7;8). AerC is a soluble chemoreceptor that functions as an energy taxis transducer by monitoring changes in redox status via FAD cofactor binding with its PAS domain (7). Similarly, Tlp1 functions as an energy taxis transducer. Tlp1 protein domain organization includes an N-terminal periplasmic region, that

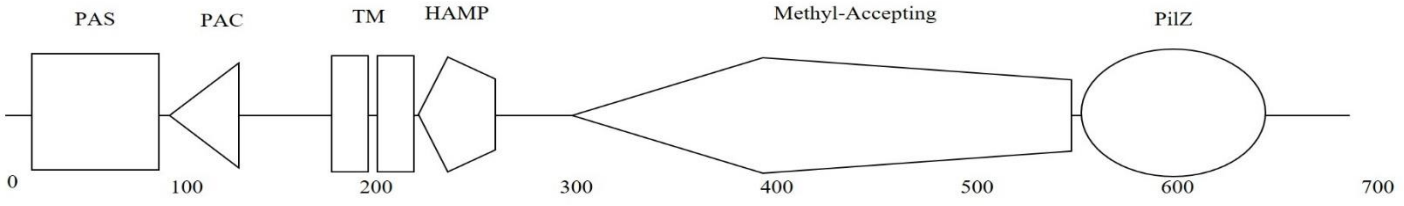

Figure 1: Characterization of Aer protein domain architecture, containing a Per-Arnt-Sim (PAS) domain, PAC domain, Transmembrane (TM) domains, Histidine kinases, adenylate cyclases, methyl accepting proteins and phosphatases (HAMP) domain, Methyl accepting (MA), and PilZ domain. N-terminal of protein is located at the end near the PAS domain and C-terminal is located on the end near PilZ.

contains a sensory domain of unknown specificity, a cytoplasmic C-terminal signaling module, and an additional C-terminal PilZ domain, which is shown to bind c-di-GMP and control sensitivity to oxygen changes (9). While Tlp1 modulates sensitivity in a dynamic oxygen gradient, it also is indispensable for plant root colonization (8).

Root surface colonization which is predicated on chemotaxis is a prerequisite for the bacterium-mediated plant growth promoting. The most robust form of chemotaxis in *A. brasilense* is aerotaxis (movement through an oxygen gradient) (13). Our lab has identified a transmembrane receptor known as Aer which contains a PAS domain, two transmembrane domains, HAMP and MA, and a PilZ domain (**Figure 1)**. In the following, we will characterize the Aer receptor, specifically its role in aerotaxis, sensing reactive oxygen species and root exudates, and indispensability in plant root colonization.

Results

Aer modulates aerotaxis and PilZ Domain is required for signaling

Aer is a prototypical chemoreceptor containing a ligand binding domain, HAMP domain, and methyl accepting domain **(Figure 1).** It also contains an additional domain, the PilZ, at the C-terminus. Previous work in this lab has shown that the PilZ domain is important in responding to oxygen gradients in addition to the observation that aerotaxis is the strongest behavioral response in this species (13). As a result, we predicted that Aer would play a role in sensing oxygen gradients. To assess the efficacy of Aer as an aerotaxis receptor, we utilized a spatial aerotaxis oxygen gradient assay comparing the behavior among the wild type strain, WT- (empty vector), markerless deletion mutant, Δ*aer*- (empty vector) and the complemented strains, full length Δ*aer*- (Aer) and truncated Δ*aer*- (AerΔPilZ). In this aerotaxis assay, a capillary tube is filled with motile cells, and equilibrated with N_2. Then, an oxygen gradient is established via diffusion of air from the atmosphere into the capillary tube containing the suspended cells. Under these conditions, the WT- (empty vector) and Δ*aer*- (Aer) strains accumulate at a region corresponding to the optimum oxygen concentration needed for growth (**Figure 2)**. In addition, our observations from the cells expressing Δ*aer*- (empty vector) were still able to produce a sustained accumulation, but at a lower oxygen concentration compared to WT (empty vector), suggesting a role for Aer in locating optimum oxygen concentration within a gradient. Similar behavior between WT- (empty vector) and Δ*aer*- (Aer) indicates the defect seen in the full deletion mutant, Δ*aer*- (empty vector), is solely due to the lack of Aer. Cells expressing Δ*aer*- (AerΔPilZ) formed a band closer to the liquid-air interface relative to the wild type (**Figure 2)**. This observation conveys the importance of a functional PilZ domain in locating the optimum position in the gradient. We also observed that this accumulation close to the air-liquid interface was not sustainable as cells began to clump together and eventually lost motility. When *A. brasilense* cells are exposed to elevated levels of oxygen, the cells adopt a response to form transient clumps; cell-to-cell clumping is a sort of "metabolic scavenging strategy" that prepares cells for further metabolic stress (10). Thus, these additional observations indicate that the PilZ domain is necessary to position *A. brasilense* in optimum, microaerophilic conditions. Overall, this assay

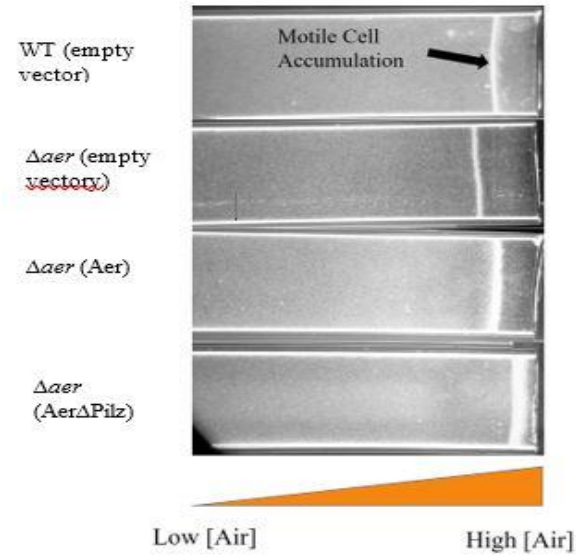

Figure 1: Spatial gradient aerotaxis assay under conditions of Nitrogen (N_2) fixation. Cells are equilibrated with nitrogen (N_2). Thereafter an oxygen gradient is established via diffusion. Representative images of WT with mutant derivates are shown. Arrow indicates accumulation of motile cells in WT sample.

demonstrated that both full length Aer and the PilZ domain are important in modulating an aerotaxis response.

Δ*aer*- (empty vector*)* displays altered chemotactic response to wheat roots

To probe the influence Aer has in the response *A. brasilense* exhibits in the chemical environment exuded by wheat roots, we performed a root in pool assay (See "Material and Methods") to identify the short-term bacterial response in a complex gradient of root exudates. In this assay, when exposed to the wheat roots, WT-(empty vector) accumulated in tight bands away from the root hairs and at 100μm away from the root tip, creating a zone of depletion (**Figure 3**). In contrast, the strain lacking the full-length Aer, Δ*aer*- (empty vector), exhibited non-specific accumulation away from the root hair and elongation zones, that were different from WT. Unlike the WT, Δ*aer*- (empty vector) was not at all repelled from the root tip (**Figure 3**). Complementing the deletion mutant with full length Aer did not restore function in the root hair zone and elongation zone; however, it did partially restore proper functioning near the root tip as cells migrated away from this zone (**Figure 3**). The Δ*aer*- (AerΔPilZ) strain was unable to accumulate around the root hair zone, elongation zone, or root tip, forming homogenous pool of cells at each location. Lacking the PilZ suppressed the ability for the cells to chemically sense the environment exuded by the roots.

Cells lacking PilZ are defective in wheat root colonization

Given the data from the aerotaxis assay, we sought to examine how this response contributed to the colonization of wheat root seedlings. Root associated microorganisms such as *A. brasilense* improve plant growth and to be able to confer these benefits, it must be able to establish itself on the roots' surfaces (14). Our methodology consisted of inoculating wheat seedlings with the different strains and to measure root surface colonization over five days post inoculation; bacteria that had colonized root surfaces were recovered by homogenizing the roots in buffer, performing serial dilutions followed by plating to determine colony forming units (CFU) via direct counting. The Δ*aer*- (empty vector) showed similar levels of root colonization as the WT- (empty vector), when inoculated alone (**Figure 4)**. In addition, the restored complement, Δ*aer*- *(*Aer), colonized at greater levels compared to WT- (empty vector) (**Figure 4)**. The strain lacking PilZ receptor was unable to colonize the wheat roots (**Figure 4)**. These findings indicate that Aer does not affect wheat root colonization. We also conducted a competition-based root colonization in which we inoculated equal ratios of both WT- (empty vector) and Δ*aer*- (empty vector). After 5 days, we homogenized the roots and recovered a ratio of WT (empty vector) to Δ*aer* (empty vector) 92:8. This demonstrates that while lack of Aer does not affect the ability of cells to colonize wheat roots, it does cause the cells to be less competitive in the rhizosphere relative to the wild type.

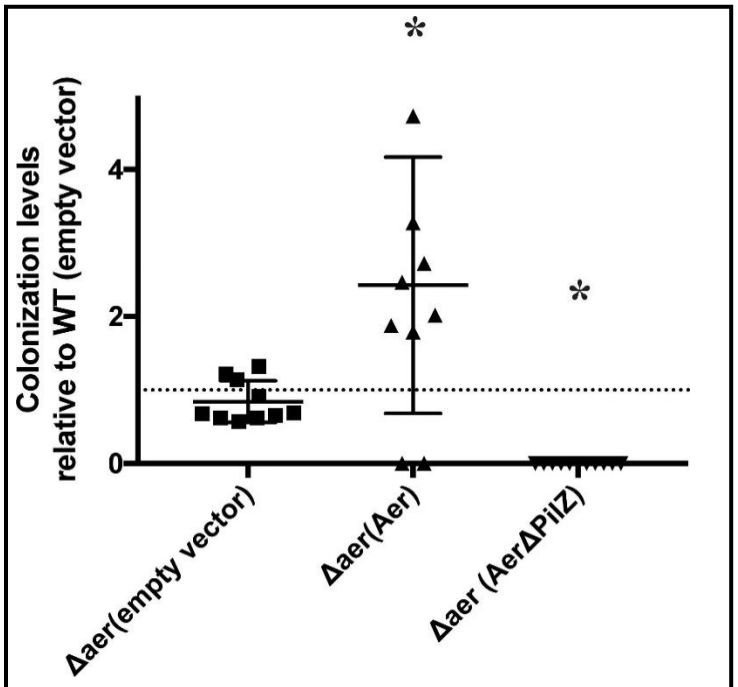

Figure 4: Colonization levels of wheat seedling roots by *A. brasilense* wild type and mutant strain derivatives. The colonization index was the $\log_{10}$(strain output/strain input), where the number of CFU extracted from roots after incubation was normalized to the number of CFU measured in the inoculated input. The dotted line value at 1 indicates the colonization index of the WT (empty vector). Positions above the line represent higher colonization efficiency, while positions below the line are indicative of lower colonization efficiency. * indicates significant difference from WT (empty vector) (p-value < 0.05).

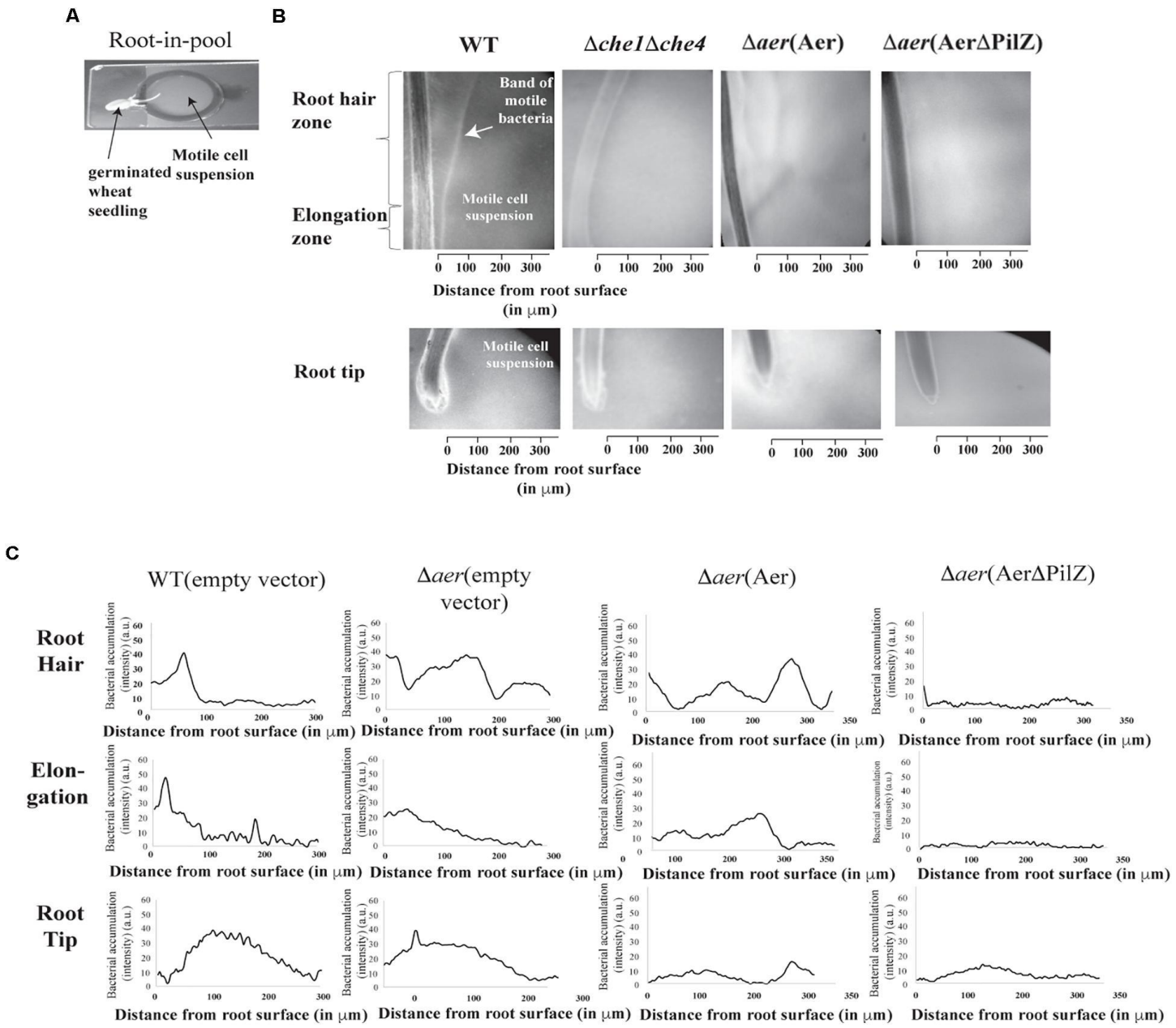

Figure 3: A. brasilense response accumulation exhibited in chemical environment exuded by wheat root exudates using a root in pool assay. (A) Apparatus constructed allows monitoring of bacterial response. (B) Bacterial cell accumulation images (recorded by Nikon Coolpix digital camera). (C) Intensity plots of bacterial accumulation.

Cells lacking full length Aer lack the ability to sense oxidative stress

Because the root tip is a zone reported to release reactive oxygen species (ROS) as it grows (1) and the Δ*aer*- (empty vector) did not migrate away from this region while the WT(empty vector) did, we hypothesized that the full length Aer aids in the ability to sense oxidative stress. To test this hypothesis, we performed a ROS disc assay. To test responsiveness to oxidative stress, WT and Δ*aer*- (empty vector) were exposed to two types of oxidative stress. In this disc assay, filter paper soaked in the oxidative stressor, either H_2O_2 (3%) or H_2O_2 (0.3%), was placed on a bacteria-soft agar mix. Response was observed for 4 hours. While the WT-(empty vector) responded to the ROS in a concentration dependent manner, the Δ*aer*- (empty vector) did not (**Figure 5)**. Cells expressing Aer in the Δ*aer* background (Δ*aer*- (Aer)) partially restored the ability to respond to the ROS stress in a concentration-dependent manner. In contrast, Δ*aer*- (AerΔPilz) displays an altered response as evidenced

by a non-concentric circle around the ROS discs (**Figure 5)**, suggesting the lack of PilZ affect the ability of cells to properly respond to the gradient (*Arrows indicate ring formation from motile cells).

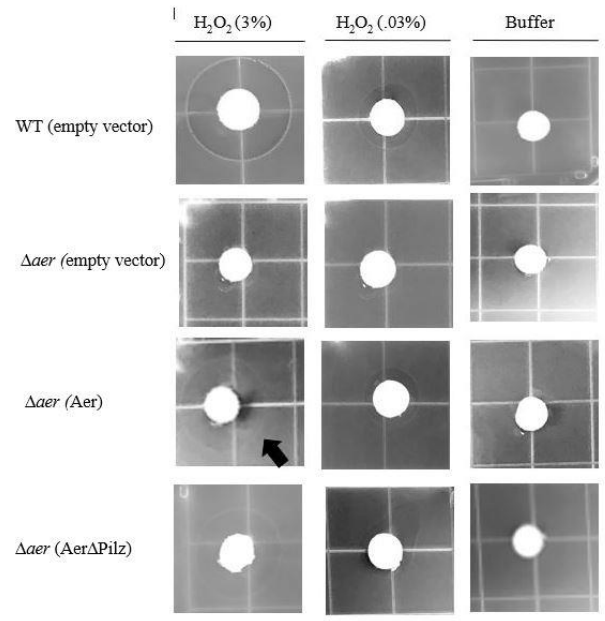

Figure 5: Reactive Oxygen Species (ROS) disc assay performed on WT and mutant derivatives, using buffer, 0.03% H_2O_2, 0.3% H_2O_2 to evaluate stress response. Bacteria were grown on soft agar mix and paper discs were soaked in appropriate reagent before applied on plate. Arrow indicates movement in a concentration dependent manner.

Discussion

Evidence from the spatial gradient aerotaxis assay suggest that full length Aer is important to *A. brasilense's* ability to locate the optimal oxygen concentration for metabolism. Strains expressing Δ*aer*- (AerΔPilz) formed tight bands at higher oxygen concentrations, where the cells experienced high stress, suggests the PilZ domain is important in mediating position of cells to lower oxygen concentrations. While Aer was not essential for wheat root colonization, it was necessary to sense chemicals exuded by root exudates, specifically reactive oxygen species. In addition, results from the root in pool assay suggest the PilZ domain plays a critical role in ensuring chemotaxis signaling function.

Many of the effects we observed may be attributed to the chemotaxis signal integration within *A. brasilense.* Universally, bacterial chemotaxis arrays are composed of MCP's associated into trimers-of-receptor-dimers coupled to CheA P5 domains and CheW to form hexagonal lattices that extend across the membrane. This architecture is likely responsible for the cooperativity among different chemoreceptors (11). In the absence of full-length Aer in the spatial gradient aerotaxis assay, the cells were still able to accumulate, suggesting other chemotaxis receptors were involved in mediating a response. Further evidence of this complex process is exhibited in the wheat root colonization assay, in which the strain expressing Δ*aer*- (empty vector) was still able to colonize the wheat seedlings at a comparable level to the WT, albeit at a far less competitive level. Additional evidence presented here alludes that Aer, specifically the PilZ domain aids in signal integration. Revisiting the spatial gradient aerotaxis assay, the cells in the truncated complement lacking the PilZ domain cannot sustain an accumulation of cells indicating the PilZ domain is important in modulating taxis in an oxygen gradient. Additionally, the same cell line was unable to colonize the wheat roots, also suggesting that the PilZ domain is crucial in modulating taxis in a complex chemical gradient. Overall, these finding suggest the importance of PilZ in Aer in mediating chemotaxis.

The aerotaxis and chemotaxis receptor, Tlp1, binds c-di-GMP via its C-terminal PilZ domain and promotes persistent motility by increasing swimming velocity and decreasing swimming reversal frequency, which helps A. brasilense reach low-oxygen zones (9). Moreover, given that oxygen and chemical gradients affect the redox status of the cell, specifically ROS severely altering redox homeostasis (12) , the assays performed here help delineate Aer's role as "redox sensor", specifically in binding c-di-GMP via PilZ to integrate redox cues such as detection of ROS species.

While Aer is not indispensable in wheat root colonization, it is critical for competitive root colonization. The findings presented here elucidate the importance of the impact of Aer on chemotaxis cell signaling integration and c-di-GMP signaling in *A. brasilense*'s ability to detect chemical gradients in the complex chemical environment presented in the rhizosphere.

Materials and Methods

Spatial Gradient Aerotaxis Assay

Azospirillum brasilense strains were grown on MMAB to OD_{600}= 0.8. One mL of culture was centrifuged and washed with Che buffer (1.7g L^{-1} dipotassium phosphate, 1.36g L^{-1} monopotassium phosphate, 0.1mM EDTA). Capillary tube was then filled with washed cells and equilibrated with Nitrogen (N_2) for 3 minutes. After Nitrogen (N_2) was turned off, air was introduced to establish a gradient in the cell suspension. Band formation of motile cells was monitored with a Nikon E200 microscope and recorded with a Nikon Coolpix digital camera. Distance of band formation was measured with FIJI program.

Root in Pool Assay

After seeds are washed with bleach, 70% ethanol containing Triton, and sterile water, they are left to germinate in the dark for 2 days. Following 2 days, the seeds are exposed to light and the roots are allowed to grow. The apparatus is a depression slide outfitted with an O-ring with a slit cut in it. The germinated root is placed into the slit of the O-ring. The O-ring is then filled with washed bacteria (Che-buffer 3X). A cover slip is applied, and imaging is started.

Plant Root Colonization Assay

Seeds were covered with bleach and rinsed with deionized water. Seeds were then covered with 70% ethanol containing Triton and rinsed again with deionized water. Seeds were then plated with water and allowed to germinate in the dark for two days. After two days, seeds were allowed to germinate in the light for one day. Bacterial strains were grown to OD_{600} = 0.5-0.8, standardized to 2 mL, and washed and suspended in 400uL of ChE buffer (1.7g L^{-1} dipotassium phosphate, 1.36g L^{-1} monopotassium phosphate, 0.1mM EDTA). Plant growth chambers were filled with semi-solid Fahreous media. Once Fahreous semi-solid has solidified, four sterile and germinated seedlings were placed equidistant from each other in the plant growth chamber. 20uL of washed bacteria was then inserted into the center of the plant growth chamber. After five days, the plant roots were retrieved and homogenized. Serial dilutions were performed.

ROS Disc Assay

Twenty-five mL of cells were grown to OD_{600}= 0.5-0.8 in TY liquid with appropriate antibiotics. The entire culture was centrifuged (800xG for five minutes, three times), washed with Che buffer, and resuspended in 25mL of Che buffer and mixed with 25mL of TY semi-solid (0.3% agar) (1:1 v/v). Twenty-five mL of bacteria-soft agar TY was poured into square petri dished. Sterilized filter paper disks soaked in 20 microliters of 0.3%/ 0.03% hydrogen peroxide, or Che buffer (1.7g L^{-1} dipotassium phosphate, 1.36g L^{-1} monopotassium phosphate, 0.1mM EDTA) were placed on top of the bacteria-agar mixture. Chemotaxis response was monitored over the course of two hours. Response was denoted by a formation of a visible ring of bacteria away from the disc. Response was recorded with a Nikon Coolpix digital camera.

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