

Root-to-Root Travel of the Beneficial Bacterium *Azospirillum brasilense*†

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The root-to-root travel of the beneficial bacterium *Azospirillum brasilense* on wheat and soybean roots in agar, sand, and light-textured soil was monitored. We used a motile wild-type (Mot⁺) strain and a motility-deficient (Mot⁻) strain which was derived from the wild-type strain. The colonization levels of inoculated roots were similar for the two strains. Mot⁺ cells moved from inoculated roots (either natural or artificial roots in agar, sand, or light-textured soil) to noninoculated roots, where they formed a band-type colonization composed of bacterial aggregates encircling a limited part of the root, regardless of the plant species. The Mot⁻ strain did not move toward noninoculated roots of either plant species and usually stayed at the inoculation site and root tips. The effect of attractants and repellents was the primary factor governing the motility of Mot⁺ cells in the presence of adequate water. We propose that interroot travel of *A. brasilense* is an essential preliminary step in the root-bacterium recognition mechanism. Bacterial motility might have a general role in getting *Azospirillum* cells to the site where firmer attachment favors colonization of the root system. *Azospirillum* travel toward plants is a nonspecific active process which is not directly dependent on nutrient deficiency but is a consequence of a nonspecific bacterial chemotaxis, influenced by the balance between attractants and possibly repellents leaked by the root.

Beneficial rhizosphere bacteria of the genus *Azospirillum* have been used as plant inoculants to improve plant growth and productivity (17, 30). When these bacteria colonize roots, their distribution is uneven (3). Although bacterial cells can be found anywhere in the root system of many plant species (23), they have a preference for the root tip, the elongation zone, and the root hair zone (18, 28, 43, 62). In the field, cells originating from soil surface inoculation can be found everywhere in the root system and as deep as 50 cm. When *Azospirillum* cells are inoculated far from plants, they can migrate horizontally in plant-free soil as far as 30 cm from the original inoculation site (13, 20). Thus, bacterial travel is an important factor in agricultural applications.

Azospirillum cells are remarkably chemotactic, both toward chemoattractants in vitro (33, 34, 47, 53, 66) and during movement through the soil toward plants (8, 14). They are also aerotactic (4) and redox-tactic (31). In soil, *Azospirillum* movement depends, at least in part, on the presence of plants because roots provide an alternative mechanism for the dispersal of cells through the soil (13). Root tips were shown to be efficient vectors for passive vertical transfer of *Azospirillum brasilense* as deep as 29 cm from the inoculation site (16). Without plants, the bacteria are rapidly and strongly adsorbed onto the clay and organic fraction of the soil, where movement is extremely restricted, even in the presence of percolating water (15). However, dispersion and colonization of *Azospirillum* cells from the inoculated seed to the root system of the plant and to adjacent plant: have yet to be explained.

The aims of this study were to explore the major mechanisms involved in the colonization of the entire root system

from inoculated seed and the way in which roots of adjacent plants are colonized. We analyzed the interroot travel of *A. brasilense* cells within the root systems of soybean and wheat seedlings by comparing highly motile wild-type bacteria with nonmotile mutants of the same strain in agar medium, a sand mixture, or a light-textured soil and in the presence or absence of nitrogen sources, chemoattractants, and repellents.

MATERIALS AND METHODS

Organisms and growth conditions. In this study we used *A. brasilense* Cd (ATCC 29710), which is a highly motile strain (Mot⁺), and a nonmotile spontaneous mutant (Mot⁻) derived from a previous Tn5 mutant of strain Cd (strain 29710-10b). Compared with the parental strain, strain 29710-10b is apparently defective only in N₂ fixation and aggregating ability in the soil; growth rate and other characteristics were identical to those of the parental strain (Cd), as described in detail elsewhere (21, 24). The identical antigenic characteristics of the Mot⁻ strain allowed us to lose antibodies raised against strain Cd (Mot⁺) (unpublished data) for enzyme-linked immunosorbent assay (ELISA) determination (described below). The Mot⁻ mutant was isolated by (i) evaluating its inability to swarm on solid medium surface (32, 40) and (ii) using light-microscopic preparations of bacteria from the logarithmic phase of growth or obtained from very young colonies (<24 h). The chosen mutant was completely nonmotile for several hours as analyzed by an image analysis system. Therefore, the mutant used in this study can be characterized as Nif⁺ Agg⁻ Mot⁻.

Wheat plants (*Triticum aestivum* cv. Tikal) (winter wheat) and soybean plants (*Glycine mar* cv. Pella) were used as test species. Seeds were surface disinfected with 1 % NaOCl for 5 min and then thoroughly washed with sterile deionized water. The seeds were soaked for 5 h in sterile tap water prior to transfer to the agar-glass assay chambers (15 by 20 by 1 cm: containing two plants each, one in each compartment), as

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† This study is dedicated to the memory of the late Avner Bashan.

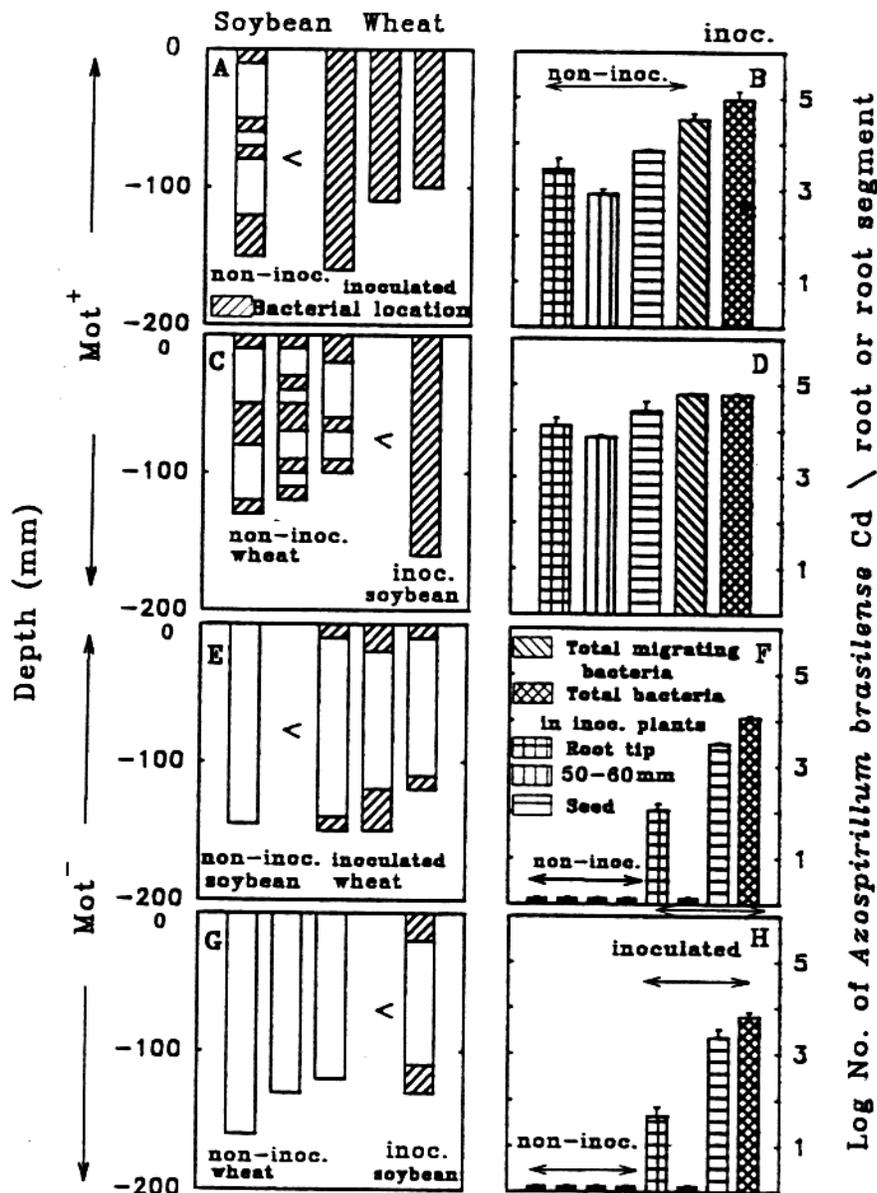


FIG. 3. Travel of *A. brasilense* Mot⁺ (A to D) and Mot⁻ (E to H) cells from inoculated (inoc) roots to noninoculated (non-inoc.) roots of different plant species in soft agar. (A and E) Travel from inoculated wheat roots to noninoculated soybean roots. (C and G) Travel from inoculated soybean roots to noninoculated wheat roots. (B, D, F, and H) Number of bacteria on different zones of the root. Error bars represent standard error, and the 0-mm point represents the seed site in the growth chamber. In panels F and H, the empty columns on the left represent the absence of migrating bacteria in segments of noninoculated roots (similar to the segments in Fig. 2). Other empty columns in inoculated roots represent the absence of bacteria in segments located 50 to 60 mm from the seed site. Bullet arrows (←) represent the expected travel direction.

stereoscopic microscopy of every colony that developed on the nutrient agar plates.

Chemoattractants, repellents, and nitrogen sources for *A. brasilense*. We used the chemoattractants glycine (10 mM), aspartic acid (10 mM), sodium malate (100 mM), and sodium succinate (100 mM) (53, 67); the repellents *p*-nitrophenylglycerol (0.006%) and NaEDTA (0.17%) (32); and the nitrogen sources KNO₃ and NH₄Cl (10 mM) (4), all of analytical grade. These chemicals were embedded in alginate beads or applied directly to the roots as described below.

Alginate bead chains as ARs. Bacteria (2×10^7 to 3×10^7 CFU/g [fresh weight] of beads) and chemicals were separately entrapped in solid, sterile, alginate beads (diameter, 3 to 4

mm) produced as described previously (6). By using a hypodermic needle, the beads were aseptically and carefully threaded onto a very fine nylon thread (diameter, 175 ± 20 μ m) in strings of up to 15 cm long. These strings of beads, or artificial roots (ARs), simulated inoculated roots (beads with bacteria) or noninoculated roots (beads with chemoattractants, repellents, or nitrogen sources) and were embedded in the agar compartments as if they were plants. Chemotaxis of *A. brasilense* toward sodium alginate was tested by the classic capillary test for bacteria in general (1) and the open-channel chemotaxis system developed for *Azospirillum* cells (4). We observed that alginate was not a chemoattractant for *A. brasilense*.

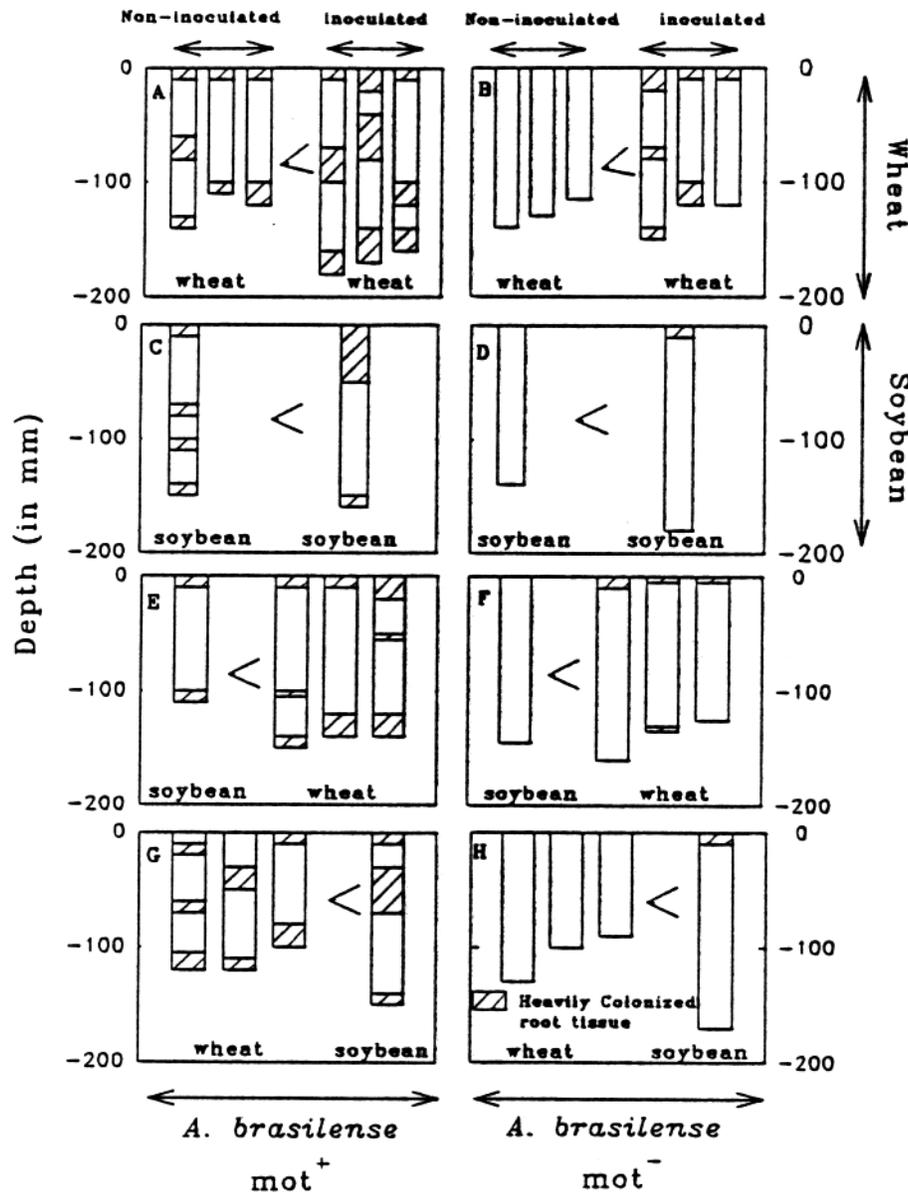


FIG. 4. Travel of *A. brasilense* Mot⁺ (A, C, E, and G) and Mot⁻ (B, D, F, and H) cells from inoculated roots to noninoculated roots of the same plant species and to different plant species in sand. (A and C) Travel from inoculated roots to noninoculated roots of wheat and soybean, respectively. (E and G) Travel from inoculated wheat roots to noninoculated soybean roots and from inoculated soybean roots to noninoculated wheat roots, respectively. (B and D) Travel from inoculated roots noninoculated roots of wheat and soybean, respectively. (F and H) Travel from inoculated wheat roots to noninoculated soybean roots and from inoculated soybean roots to noninoculated wheat roots, respectively. The 0-mm point represents the seed sites in the assay chamber. Bullet arrows (←) represent the expected travel direction.

Oxygen and nitrogen measurements. The oxygen concentration within the chambers was measured with an oxygen electrode, and the nitrogen content of some ARs was measured by using the micro-Kjeldahl method (for NH₄Cl) and by the nitrate determination method of Strickland and Parsons (58) (for KNO₃).

Experimental design, statistical analysis, and data presentation. All treatments were replicated three times (one assay chamber as a replicate), and each experiment was repeated two or three times. Significance is given by the standard error for each column. Bacterial counts were taken from the average of similar root sections from three different seedlings and were

made independently of the spatial distribution maps of the bacteria in the roots.

The wheat root system of seedlings consisted of three main roots; the soybean root system of young seedlings consisted of a single root. The graphic column representation of inoculated and noninoculated roots is a substitute for color photographs, which did not show areas of colonization clearly when printed in black and white. The graphic representation of the spatial distribution of bacteria on the roots shows drawn bands; each represents the range of zones of overlapping colonization for three experiments.

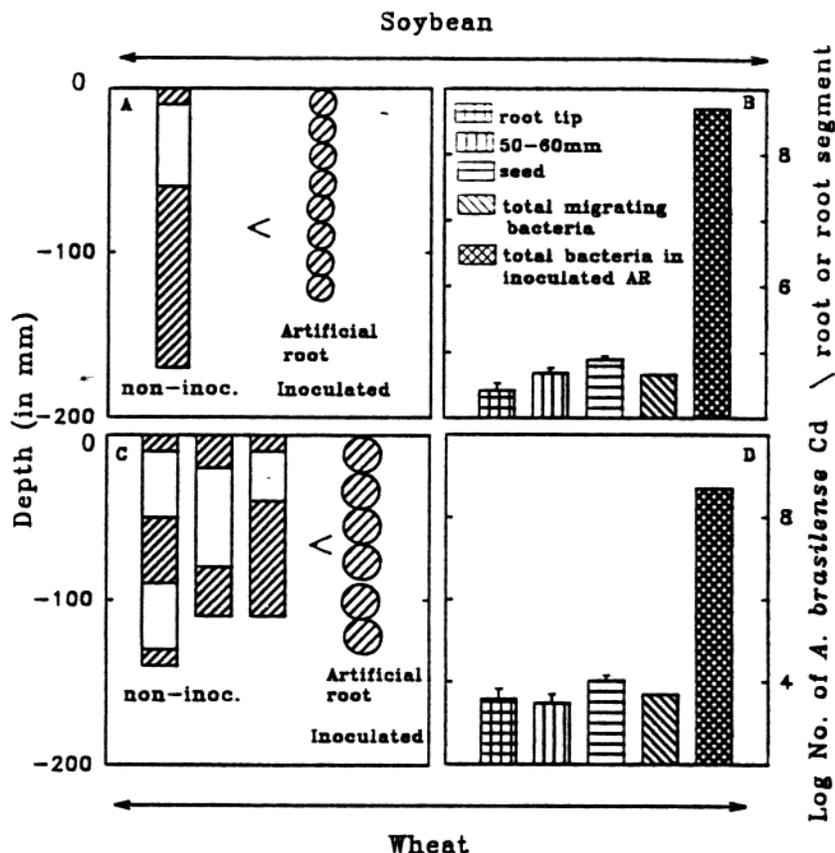


FIG. 5. (A and C) Travel of *A. brasilense* Mot⁻ cells from inoculated ARs to noninoculated (non-inoc) soybean and wheat roots, respectively. (B and D) Level of colonization of noninoculated roots of soybean and wheat, respectively. Error bars represent standard error, the 0-mm point represents the seed site in the assay chamber. Circles are only schematic and do not represent the actual size of the beads. Bullet arrows (←) represent the expected travel direction. ZZZZ, bacterial location.

RESULTS

Movement of *A. brasilense* Mot⁺ and Mot⁻ strains from inoculated roots to noninoculated roots of the same plant species and between different species. In assay chambers, *A. brasilense* Mot⁺ cells migrated for several centimeters from inoculated roots to the noninoculated roots of the adjacent plant, irrespective of the plant species. Colonization of the noninoculated roots was band type: i.e., most bacteria were concentrated in defined root zones, and few were detected in the rest of the root. The inoculated roots were entirely colonized (Fig. 2A and C). Colonization of the noninoculated (target) wheat roots was significantly lower than colonization of the original inoculated root (Fig. 2B). In contrast to noninoculated wheat, noninoculated soybean roots were colonized to the same extent as inoculated roots were (Fig. 2D). Root tips and seed sites were always colonized by *A. brasilense* Mot⁺ cells (Fig. 2A and C).

A different pattern was detected for the *A. brasilense* Mot⁻ mutant. Bacteria did not move from the inoculated roots to the noninoculated roots (Fig. 2E and G). The pattern of colonization in the seed-inoculated roots was also different, because bacteria were concentrated either near the inoculated seed or in the root tip of wheat (Fig. 2E to H). In both plant species, the total level of colonization of the original inoculated roots by the Mot⁺ and Mot⁻ strains was high (Fig. 2B, D, F, and H).

Similar patterns were observed when movement between soybean and wheat roots was monitored. *A. brasilense* Mot⁺

cells moved from inoculated wheat to noninoculated soybean and vice versa (Fig. 3A to D). *A. brasilense* Mot⁻ cells did not move from inoculated to noninoculated roots, regardless of the plant species (Fig. 3E to H). The level of colonization of noninoculated roots as a result of bacteria migrating between the plant species was similar to the level detected when bacteria were migrating between plants of the same species (Fig. 3B, D, F, and H).

In sand, *A. brasilense* Mot⁺ cells moved between the roots of the same species or different species, similar to their pattern in agar (Fig. 4A, C, E, and G). No movement of *A. brasilense* Mot⁻ cells from inoculated to noninoculated roots was detected. In many cases, the inoculated bacteria remained at the seed inoculation site and did not migrate with the root tips (Fig. 4B, D, F, and H). The population levels of bacteria on roots in sand were similar to those in agar (data not shown). Motility in the light-textured soil was similar to that in sand (data not shown).

Movement of *A. brasilense* Mot⁺ cells from inoculated ARs to noninoculated wheat and soybean roots. When ARs were embedded with *A. brasilense* Mot⁺ cells and exposed to natural noninoculated roots of either wheat or soybean, *A. brasilense* Mot⁺ cells moved from the ARs to the target noninoculated roots (Fig.

Movement of *A. brasilense* Mot⁺ and Mot⁻ cells from inoculated roots toward chemoattractants and repellents. When an AR containing the chemoattractant glycine was

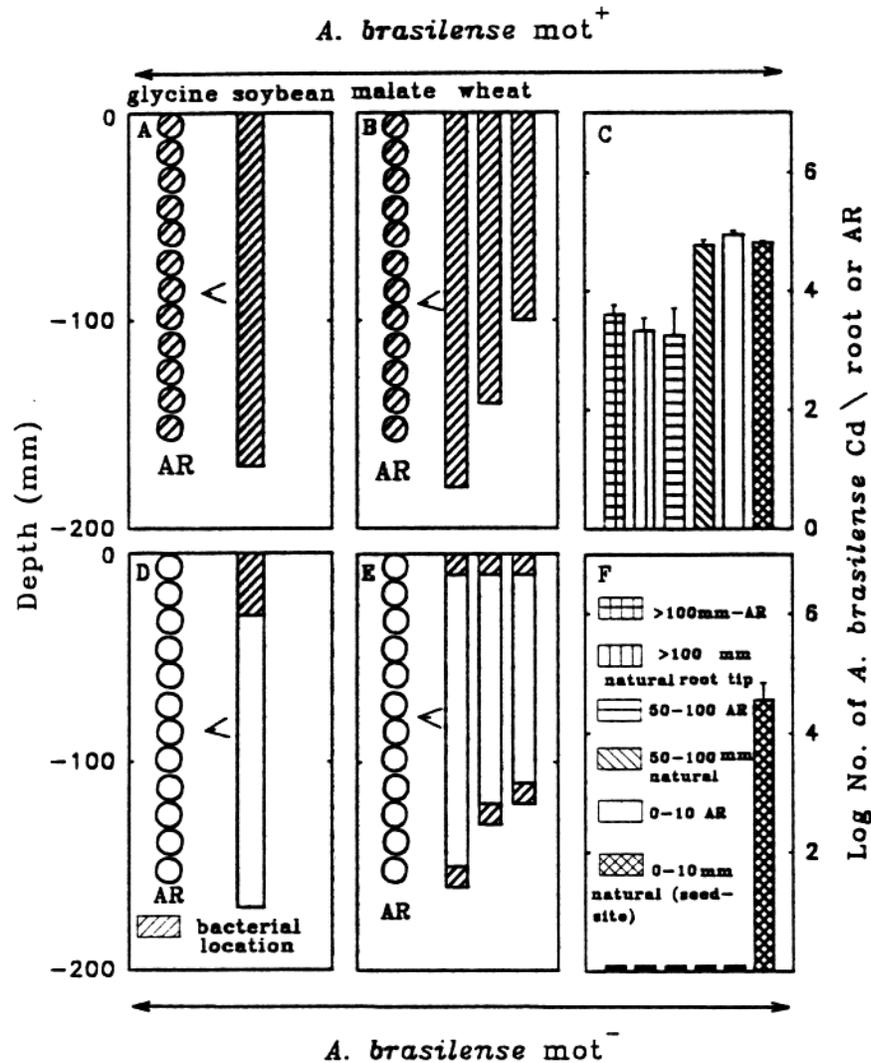


FIG. 6. Travel of *A. brasilense* Mot⁺ (A to C) and Mot⁻ (D to F) cells from inoculated roots toward chemoattractants trapped in ARs. Error bars represent standard error, and the 0-mm point represents the seed site in the assay chamber. Circles are only schematic and do not represent the actual size of the beads. Bullet arrows (←) represent the expected travel direction.

placed in the same assay chamber in the opposite compartment relative to inoculated soybean roots, *A. brasilense* Mot⁺ cells migrated toward the AR (Fig. 6A). The same was true when an AR containing malate was placed with inoculated wheat roots (Fig. 6B). Similar results were also obtained with the combinations glycine-wheat and malate-soybean (data not shown). When *A. brasilense* Mot⁻ cells were used, no migration toward an AR containing glycine was observed in soybean (Fig. 6D) or the malate-wheat combination (Fig. 6E). *A. brasilense* Mot⁺ cells colonized the AR in the same numbers as it colonized inoculated roots (Fig. 6C). *A. brasilense* Mot⁻ cells were found only in the seed site of inoculated roots (Fig. 6F).

When the attractants were placed on restricted zones of the AR (arrows in Fig. 7A to D), *A. brasilense* Mot⁺ cells migrated from the inoculated roots to the attractant sites. Similar results were obtained with the combinations glycine-soybean (Fig. 7A), malate-soybean (Fig. 7B), and malate-wheat (Fig. 7D) and when combinations of three attractants (aspartate, succinate and malate) were used in an AR placed in the assay chamber with inoculated soybean root (Fig. 7C).

When ARs loaded with the repellent *p*-nitrophenylglycerol or NaEDTA were placed in the same assay chamber in the opposite compartment relative to inoculated soybean roots, *A. brasilense* Mot⁺ cells did not migrate toward the AR (Fig. 8A and b). Noninoculated soybean roots, when loaded with the same repellents, also repelled *A. brasilense* Mot⁺ cells along the root, except at the root tip zone (Fig. 8C and D).

When the AR was loaded segmentally with a repellent (NaEDTA) and an attractant (malate), *A. brasilense* Mot⁺ cells migrated to the attractant but not to the repellent (Fig. 8E), even if these zones were only 3 to 4 mm long (one bead) (Fig. 8F).

Movement of *A. brasilense* Mot⁺ cells from an AR containing nitrogen sources to an AR containing attractant. Movement of *A. brasilense* Mot⁺ cells from the AR containing the nitrogen source KNO₃ to an AR containing an attractant was monitored over 72 h. We found that although movement began almost immediately, it increased 48 h after inoculation, when about half of the nitrogen source was exhausted (Fig. 9B). At 72 h after inoculation, 99.98% of all bacteria in the assay chamber

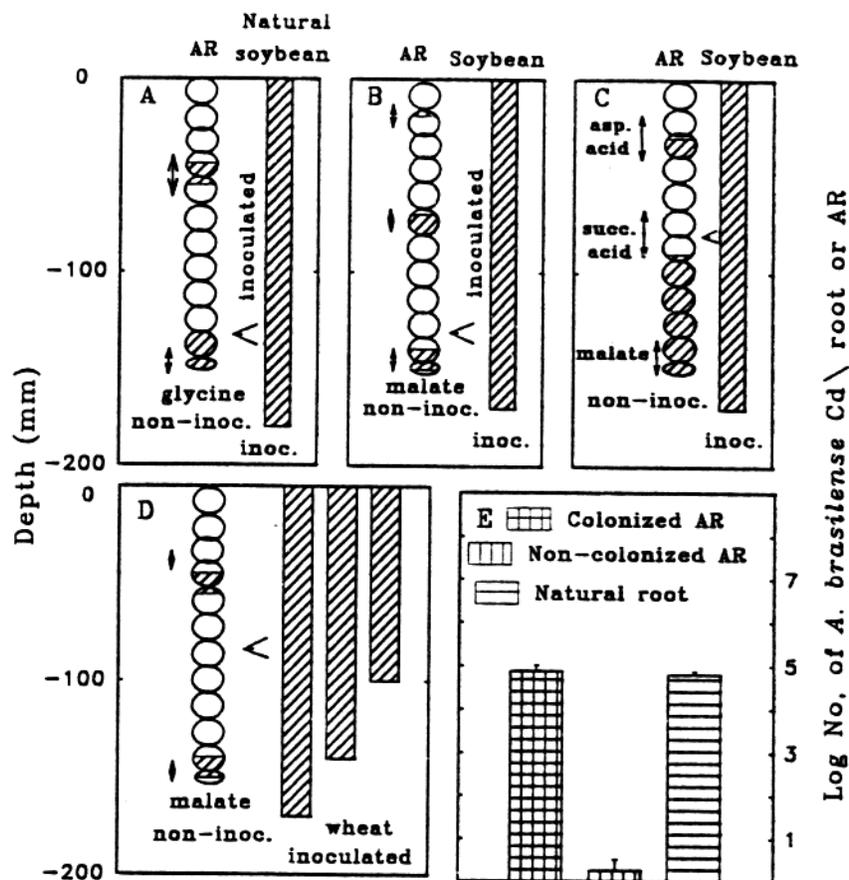


FIG. 7. Travel of *A. brasiliense* Mot⁺ cells from inoculated roots toward chemoattractants located in restricted zones in AR (arrows \updownarrow). Error bars represent standard error, and the 0-mm point represents the seed site in the assay chamber. Circles are only schematic and do not represent the actual size of the beads. Bullet arrows (\leftarrow) represent the expected travel direction. ||||| , bacterial location. inoc., inoculated; non-inoc., noninoculated.

were located in the AR containing the attractant (Fig. 9A). Similar results were obtained when NH_4Cl and malate or aspartate were used (data not shown).

DISCUSSION

To increase plant productivity, root colonization by beneficial bacteria may be a fundamental requirement (17, 25, 39). Parke (50) proposed that the colonization process begins with a dispersal phase resulting from artificial inoculation of either the root or seed surfaces, active movement of the bacteria toward the root, or random contact of the growing root with bacteria in the soil. Only scattered reports of these processes can be found in the literature, except for reports on movement of rhizobia and genetically altered pseudomonads in the soil and rhizosphere (37, 45, 61).

Azospirillum species are known to be good root colonizers of many cereal species in tropical and semiarid environments (17). In addition, the bacteria can colonize numerous non-cereal plant species (23), alginate beads (6), γ -irradiated dead roots (18), and polystyrene surfaces (10). However, under temperate conditions, their numbers decline rapidly compared with those of beneficial pseudomonads. *Bacillus* species, and other saprophytic bacteria (17). *Azospirillum* species, which are known to be plant growth-promoting rhizobacteria, have increased the yield of many crop plants, not by the suppression of

any plant pathogens but by directly affecting plant growth through a mechanism which is not yet understood.

For practical reasons, *Azospirillum* cells are usually incorporated into inoculant carriers composed of peat, vermiculite (30, 57), or alginate (6). These inoculants are later mixed with the seeds before sowing in the field or applied to the soil during seedling emergence (20). This common inoculation technology is usually carried out by standard agromechanical sowing devices, which are inaccurate from a microbial standpoint, i.e., unable to ensure that the bacteria will encounter the emerging root. Thus, bacterial movement from the inoculation site to the root site is essential if root colonization is to occur. This distance can range from a few micrometers to several centimeters, and bacterial movement must occur in an environment of fierce competition with other soil microorganisms which are also seeking nutrients and root colonization sites on the growing seedlings (5).

Root tip growth (>1 cm/day in many plant species) is too rapid for bacterial targeting (50); therefore, root tip colonization occurs mainly by random physical encounters (16). The root elongation zone and the root hair zone are the main sites for bacterial activity in general and for *Azospirillum* activity in particular (19, 62). Thus, self-motility of plant growth-promoting rhizobacteria can be considered an important trait for rhizobacteria (49). *Azospirillum* and pseudomonad biocontrol agents have been known to colonize the entire root system of

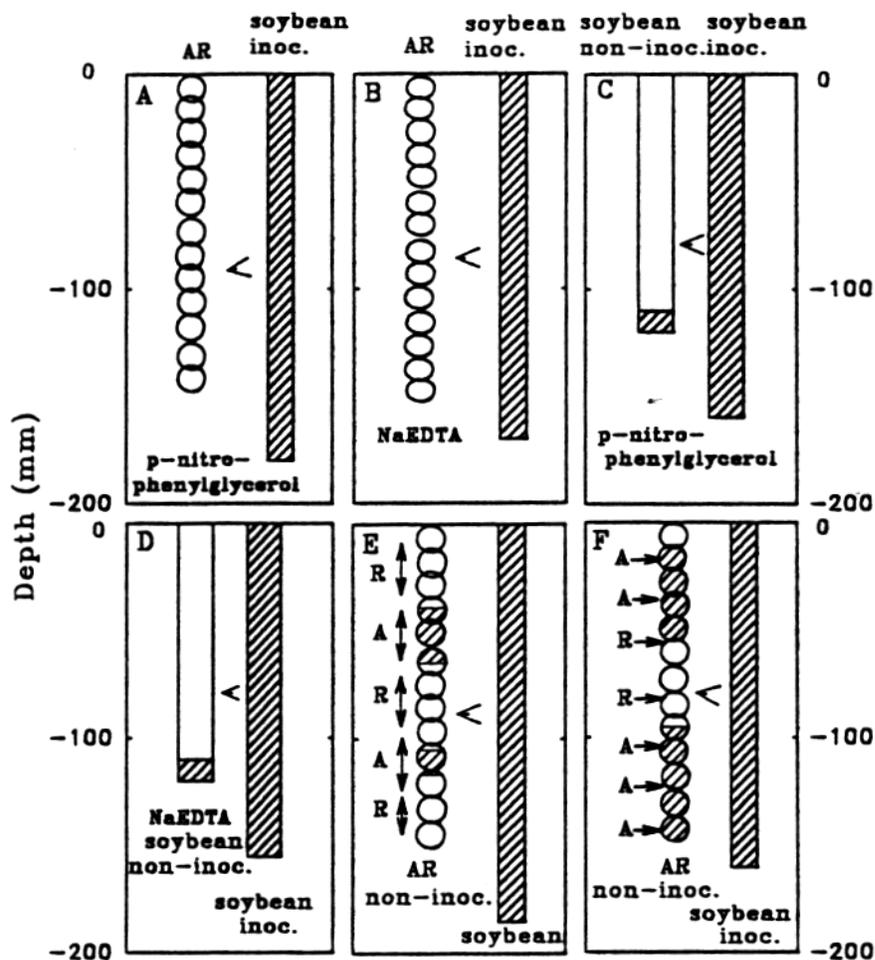


FIG. 8. Travel of *A. brasilense* Mot⁺ cells from inoculated soybean roots toward chemoattractants or repellents located in AR (A acid B) or natural soybean root (C and D) or restricted areas in AR (E and F). Abbreviations: A, attractant (malate); R repellent (NaEDTA). Circles are only schematic and do not represent the actual sine of the beads. Bullet arrows (←) represent the expected travel direction. , bacterial location. inoc., inoculated; non-inoc., noninoculated.

many plants species (19, 23); this bacterial dispersion has been partially attributed to passive transport by the root tips (16, 35).

In contrast to some pseudomonads, *Azospirillum* cells do not disperse with percolating water (2, 27, 44, 45, 51, 61, 63-65) but, rather, adsorb onto the soil particles (14, 15, 59). Nevertheless, passive dispersion by water, especially in semiarid conditions which lack sufficient water (where *Azospirillum* cells showed their best performance [54]) cannot explain how the entire root system is colonized by *Azospirillum* cells. Therefore, it is reasonable to assume that another efficient bacterial dispersion mechanism exists. The aim of this study was to reveal the major component of this mechanism, since dispersal has been suggested to be a crucial parameter in the deliberate inoculation of genetically engineered beneficial bacteria (9, 60).

This study revealed that motility of *A. brasilense* in the rhizosphere was essential to colonization of the root system. Although the nonmotile mutants proliferated similar to the wild type, they failed to colonize neighboring roots, even though water for the diffusion process was always available. Random contact of growing roots and bacteria in the soil or contact between roots harboring bacteria and bacterium-free

roots could allow for a broader form of colonization. For simplicity, mgt of the study was conducted with roots in agar trays since the basic migration profile was similar in agar trays, sand, and light-textured soil. Our results with *A. brasilense* further support studies which show that nonmotile mutants of a beneficial pseudomonad were impaired in their ability to move toward seeds (55) or colonize roots (29) compared with their wild-type parent strains.

Azospirillum cells have previously shown chemotaxis toward several compounds in vitro (38, 46, 53, 67), and this was also the case in this study. The bacteria have shown a general attraction to nonspecific chemoattractants commonly found in root exudates and to which many rhizobacteria might migrate. Therefore, a nonspecific chemotaxis to root exudates may be a preliminary mechanism of the plant-bacterium interaction, operating over relatively long distances (up to several centimeters) and before other components of plant-bacterium recognition systems (adhesive materials, lectins, etc.) take over. At these distances, the specific, local root-bacterium recognition mechanism is ineffective, as shown in this study by results with bacteria that migrated from roots to ARs. Perhaps their ability to be generalists make *Azospirillum* species good root colonizers.

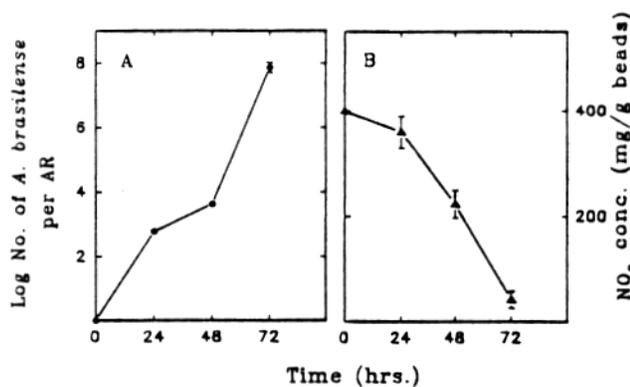


FIG. 9. Travel of *A. brasilense* Mot⁺ cells from an AR containing KNO₃ to an AR containing attractant (glycine) (A) and the level of KNO₃ in the AR (B). Bars represent standard error. Points without bars have standard errors smaller than the symbol.

We also suggest that this dispersion mechanism increases the survival of *Azospirillum* cells. *Azospirillum* cells are totally dependent on the presence of roots to survive because they survive poorly in some soils (15). The death of the host plant will not diminish the bacterial population of the field, since *Azospirillum* cells are capable of migrating to neighboring plants, whether to a different plant species (as demonstrated in this study) or to weeds under field conditions (13).

Bacterial movement to noninoculated roots from inoculated roots which were still exuding nutrients can be explained in at least two ways. (i) The bacterial population on the roots consumed more nutrients than the roots could supply at a given moment. This created a temporary nutrient-deficient microenvironmental gradient which may have stimulated migration toward alternative nutrient sources. This was demonstrated in this study by the use of artificial roots containing nitrogen sources (Fig. 9). (ii) *Azospirillum* cells undertook mainly aggregate types of colonization, which restricted cell movement because of the production of extensive fibrillar materials (19, 43). These aggregates provide an ecological advantage over any single bacterial cell in the competition for nutrients in the rhizosphere. Therefore, one can speculate that to survive, single cells must locate sites which lack aggregate colonization, so they migrate to noninoculated roots and colonize them, producing the new aggregates that were detected in this study (the pink zone on noninoculated roots, verified by microscopy). Therefore, moving to an uncolonized root may be a way in which single bacteria can effectively compete with bacterial aggregates for nutrients. A similar phenomenon was previously observed in a marine vibrio forming biofilms, in which the original cells remained attached to the surface while the progeny cells of the biofilm detached and became planktonic. These motile daughter cells colonized other surfaces to produce a new biofilm, and the cycle continued (48). The similarity between the behavior of aqueous biofilms and terrestrial bacterial aggregation is striking. One could imagine a colonization strategy that is common to some bacteria, such that aggregate colonization by *Azospirillum* cells could even be considered a rhizosphere bacterial biofilm. This terrestrial biofilm formation requires further verification under normal soil conditions and with competition from other bacteria.

Two factors affecting migration of bacteria in soils are the presence of continuous water films and the response to oxygen gradients (8, 26, 36, 62). These factors were neutralized in this

study. The systems were always water saturated and there was a continuous diffusion of oxygen inside the assay chambers. Thus, self-motility was the sole factor evaluated.

We propose that bacterial travel within the plant root system and between neighboring plants is a prerequisite in the root-bacterium recognition mechanism. Bacterial motility might have a general role in getting *Azospirillum* cells to the sites where a firmer colonization process might occur which subsequently leads to colonization of parts of the root system. This is a nonspecific mechanism. Bacterial travel is an active process and a consequence of bacterial chemotaxis influenced by the balance between attractants and possibly repellents exuded by the root.

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