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# Evidence that fibrillar anchoring is essential for *Azospirillum brasilense* Cd attachment to sand

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## Abstract

Inoculation and incubation of wild type *Azospirillum brasilense* Cd ($agg^+$) in pure quartz sand resulted in cell attachment to sand particles by a network made up of various sizes and shapes of fibrillar material. Inoculation of sand with an aggregate-deficient mutant of strain Cd ($agg^-$) resulted in no detectable fibrillar formation. Initial attachment ratio between $agg^+$ cells and $agg^-$ cells was 4:1. However, similar bacterial populations developed in the sand. Rinsing the sand, colonized by either strains, had a greater effect on $agg^-$; decreasing adsorption from 8.1 to 1.4%. Prolonged rinsing entirely desorbed its cells from the sand. Long bacterial incubation in sand decreased the attachment ratio between $agg^+$ and $agg^-$ from 3.4:1 to 2.9:1 and decreased desorption (by rinsing) from 10:1 to 6:1. Agitation increased bacterial population size (from $2 \times 10^7$ to $4 \times 10^8$ cfu $g^{-1}$) and decreased the proportion of attachment of $agg^+$ cells (from 29.2 to 9.8%). A decrease in attachment was being of higher magnitude in the non-aggregating mutant (from 5 to 0% adsorption). Protease treatment of sand colonized with either bacteria decreased attachement of $agg^+$ (from 27.4 to 7.1%) and released proteinaceous compounds) into the sand only in the $agg^+$ strain. Addition of NaEDTA to sand before inoculation, decreased attachment of $agg^+$ (from 24.2 to 14%) but had no effect on $agg^-$. Addition of low amount of clay (montmorillonite) to sand significantly increased adsorption of $agg^-$ to the sand particles (from 8.8 to 98.3%). Survival period of $agg^+$ cells in sand was slightly longer than that of $agg^-$ cells. It is proposed that bacterial fibrils are essential for anchoring of *A. brasilense* to sand.

## Introduction

The beneficial rhizosphere bacterium *Azospirillum brasilense* Cd has been applied to numerous soil types world-wide in order to inoculate crop plant roots and to enhance plant yield (Bashan and Levanony, 1990; Michiels *et al.*, 1989). In soil containing clay and organic matter, this rhizosphere bacterium shares some soil adsorption features with many other soil bacteria, *e.g.* live or dead bacterial cells are strongly and irreversibly adsorbed to soil particles; rinsing or other external physical forces have only marginal effects on bacterial adsorption (Bashan and Levanony, 1988a; Marshall, 1980).

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When *A. brasilense* Cd cells were applied to sand, which can be characterized as having small surface area, large particle size and minimal clay and organic matter content (Marshall, 1980), a relatively low level of adsorption occurred as compared to soil. However, part of the population is actively attached to sand particles by a network of protein bridging between individual cells and between cells and sand particles (Bashan and Levanony, 1988b).

The purposes of this study were to evaluate the importance of these bridges in the life cycle of the bacterium in the sand and to establish whether or not they are essential for *A. brasilense* Cd attachment to sand by comparing a mutant deficient in aggregation ability ($agg^-$) to the highly aggregated $agg^+$ wild type *A. brasilense* Cd.

## Materials and methods

### *Bacterial strains*

The following bacterial strains were used in this study: *Azospirillum brasilense* Cd (ATCC 29710; aggregating strain, $agg^+$) and a non-aggregating spontaneous mutant ($agg^-$) derived from Tn5 mutant of strain Cd {(strain 29710-10b; apparently defective only in $N_2$-fixation ability; other characteristics were identical to the parental strain (Cd) and were described in detail elsewhere (Bashan *et al.*, 1989)}. The $agg^-$ mutant was isolated as follows: *A. brasilense* 29710-10b was grown in 50 mL nutrient broth (Difco) supplemented with 1 mM NaEDTA, which partially prevents aggregation in *A. brasilense* Cd [Math, Kessel, Sadovnik and Henis, Forth International Symposium in Microbial Ecology, Ljubljana, Yugoslavia, p 119 (Abstr.)] for 5 days (200 rpm, 30 ± 2°C) forming aggregates. Aggregates were removed- by centrifugation -(800 x *g*, 5 min), 0.1 ml from the bacterial supernatant was reinoculated into fresh liquid medium and the aggregate separation procedure was repeated (six successive cultures). The last supernatant was decimally diluted and was plated on solid semi-selective BLCR medium for Azospirillum (Bashan and Levanony, 1985) supplemented with 20 m*M* $NH_4Cl$. Thirty seven colonies [dark red, <1 mm in diameter] which showed no visible production of slime (observed by stereoscopic microscope) were cultured separately in nutrient broth. Analyzing other bacterial characteristics of the $agg^-$ strain showed no significant differences from its parental strains (Cd and 29710-1Ob) (Table 1). Two bacterial cultures which showed no aggregation or fibril formation under light and scanning electron microscope, even after 10 days of continuous shaking were selected for this study.

### *Sand*

Pure silica quartz sand which contained the following materials (percentage composition) was used: fine sand, 0.2-0.6 mm, 99.7; clay (Kaolinite), 0.2; traces of organic matter and $CaCO_3$. This sand contained neither rough sand nor silt. The sand had a water field capacity of 2% (v/w) and a pH of 8.1 (Bashan and Levanony, 1988b).

### *Bacterial growth conditions and inoculation*

Both bacterial strains were cultured on nutrient broth (Difco) medium. To avoid self-aggregation of $agg^+$ cells prior to inoculation, cultures were shaken at 250 rpm for 16 h in Erlenmeyer flasks fitted with shallow grooves. This procedure allowed harvesting of non-aggregating bacteria in exponential phase of growth. Bacteria were inoculated into the sand by placing a double washed bacterial suspension in 0.06 *M* potassium phosphate buffer saline (PBS) supplemented with 0.15 *M* NaCl, pH 7.0, at a final concentration of $2 \times 10^6$ cfu per gram of sand.

### *Adsorption and desorption assays*

Before any treatment, sand was dried in a forced draught oven at 50 ± 2°C for 48 h and sterilized by *g*-irradiation (25 kGy), using a $^{60}CO$ source. The amount of solution added (bacterial culture and other solution preparations) was designed to simulate field capacity conditions of the sand.

Experiments were done in small glass Petri dishes containing 5 g dried sand. All adsorption assays contained 20 m*M* fructose and 50 m*M* $NH_4Cl$ (Bashan and Levanony, 1988b). Other substances added (in PBS) to the test assays were: NaEDTA (1 m*M*); protease (Pronase E

Table 1. Characterization of *A. brasilense* strains used in this study

| Bacterial characteristic | agg$^+$ (Cd) | 29710-106 (Nif$^-$) | 29710-10b agg$^-$ |
|---|---|---|---|
| Acetylene reduction in culture ($m$ mole $C_2H_4$/min/mg protein) | 55 | 0 | 0 |
| Nitrogenase structural genes | *nif*HDK present | *nif*HDK deleted | |
| Chemotaxis ratio towards glycine | 12.5 | 10.8 | 10.6 |
| Motility in solution | All strains are highly motile | | |
| Migration towards growing roots in sand (mm after 96 h incubation) | 75 ± 4 | 70 ± 6 | 73 ± 8 |
| Growth on minimal mineral media | + | + | + |
| Growth on N-free medium (BL) | + | - | - |
| Utilization of $NH_4^+$ and $NO_3^-$ as sole N source | + | + | + |
| Pink pigmentation | + | + | + |
| Colony morphology | Dry with protruding ridges for all strains | | |
| Aggregation at stationary phase of growth | + | + | - |
| Absorbance at 540 nm for $10^9$ cfu/ ml | 1.05 ± 0.03 | 1.05 ± 0.03 | 1.02 ± 0.04 |

Methods are described in Bashan et al. (1989).

type XIV Sigma, 1 mg g$^{-1}$, w/w, 32 ± 1°C, 60 min); denaturated protease (55 ± 2°C, 25 min) or clay (montmorillonite, 1-5% final concentration) .

Bacteria and the other solutions (listed above) were added aseptically, mixed immediately after application of bacteria, the dishes were sealed with Parafilm (to avoid drying of the mixtures) and transferred to an incubator at 30 ± 2°C for 24-48 h. Long incubation periods of bacteria in the sand were up to 25 d.

Strong agitation treatment of sand-bacteria mixtures after sand colonization (vortex mixer, Vortex-Genie, Scientific Industries) was performed at 180 rpm for 60 s before the rinsing procedure, whereas low agitation was performed at 40 rpm for 90 s. Inoculated sand was rinsed as follows: sand was placed in 50-mL beaker, and sterile tap water was added until it formed a 1 cm layer on the sand surface. Two rinsing durations were used; samples wer e stirred for 10 sec or 2 min with magnetic stirrer. Stirring was then stopped for 1 min to allow sedimentation of sand particles, supernatant was collected and the number of bacteria was determined by the improved selection technqiue on semi-selective BL medium supplemented with 20 m$M$ $NH_4Cl$ as described elsehwere (Bashan and Levanony, 1985). The remaining sand was diluted serially in sterile tap water and the minimal number of adsorbed bacteria was determined (since the dilution method diluted only sand particles, and probably more than one bacterium adsorbed to a given particle, the lowest probable number was determined). Attempts to count bacteria per sand particle by direct light microscopy observations was impractical, and it would likely be inaccurate due to the three dimensional characteristic of every sand particle.

Percentage adsorption was calculated by dividing the number of bacteria in the sediment by the total bacterial number x 100. Percentage of desorption was calculated by dividing the number of bacteria in the supernatant by the total number of bacteria x 100. Confirmation of the obtained values was made according the theoretical formula that the percentage adsorbed equals 100 minus the percentage desorbed. However, there were differences of up to 3% between the actual calculations and the theoretical value, *i.e.*, the percentage adsorption plus the percentage of desorption often equalled less than

100%. Adsorption ratios were calculated by dividing the number of adsorbed $agg^+$ cells by the number of adsorbed $agg^-$ cells.

*Bacterial enrichment*

Sand samples (0.1-0.2 g) colonized by either strain were aseptically transferred into semi-solid BL medium ($agg^+$) or to a similar medium supplemented with 20 m*M* $NH_4C1$ ($agg^-$) in test tubes and incubated motionless for 5 days at 30 ± 2°C. The typical Azospirillum pellicle formed under these conditions (for $agg^+$) and visible turbidity of the medium (for $agg^-$) were scored and confirmed on solid BL medium (Bashan and Levanony, 1985).

*Protein measurements*

Supernatant obtained after protease treatment of inoculated sand was filtered through 0.45 *m*m (Millipore) filter to remove desorbed bacteria. Inoculated sand was incubated similarly to the protease treatment in PBS and the supernatant obtained from the mixture was further treated as the supernatant obtained after protease treatment. Protein in the supernatant was measured by the Coomassie blue method (Sedmak and Grossberg, 1977). Amount of protein in the supernatant was calculated after subtracting the amount of pronase in the reaction mixture.

*Scanning electron microscopy (SEM)*

Gamma-sterilized silica quartz sand maintained in small Petri-dishes was inoculated with $10^8$ cfu $g^{-1}$ sand double washed (in PBS) of *A. brasilense* Cd ($agg^+$ and $agg^-$) in the exponential growth phase. The inoculated dishes were incubated for 24 h at 30 ± 2°C. A small amount of sand was placed in sealed sinter-glass beakers 1 x 0.8 cm inner diameter) which permitted the penetration of solutions and avoided dispersing the sand particles.

Preliminary observations showed that the following multistep procedure was required for optimal SEM preparation of sand. Beakers containing bacteria-colonized sand were immersed and fixed for 2 h in 5% (v/v) glutaraldehyde solution in 0.2 *M* PBS (pH 7.4), under vacuum and washed twice in the same buffer. Preparations were then dehydrated by passage through increasing alcohol concentrations at 4 ± 1°C. Samples were finally dried in a critical point dryer (Tousimis, USA) in freon, then in $CO_2$ atmosphere (20 min liquid $CO_2$ rinse). Dried sand was spread on adhesive tape (previously taped onto stubs) without any compaction or touching of the sand. Unattached particles were removed by a very light air stream. Samples were coated with gold (10 mAmp, 6 min, 100-150 A thickness, using S-150 sputter coater, Edwards Co. USA). A second platinum coating (50-70 Å) was applied only when necessary to reduce charging and examined by an ISI 40 Scanning Electron Microscope at 30 kV Bacteria from liquid cultures were prepared for SEM observations as follows: 3 mL bacterial culture (48 h-old) were filtered through 0.22 *m*m (Millipore) filter, and the filter was rinsed with 2 mL of double-distilled water. Bacteria on the filter were fixed for 1 h in 3% glutaraldehyde in PBS. Preparations were dehydrated as above, air-dried, coated with 100 A platinum layer and observed as above.

*Experimental design and statistical analysis*

All experiments were completely randomized design with 3 replicates, with either 3 Petri dishes, 3 tubes or 3 SEM stubs considered a single replicate. Experiments were repeated 2 to 3 times each. Results given are from one representative experiment in each case. Analysis of variance (ANOVA) was performed and significance is given at $P \leq 0.05$. Comparison between treatment means was performed by Student's t-test with significance at $P \leq 0.05$. Log of bacterial population was analyzed by ANOVA with significance at $P \leq 0.05$.

## Results

*SEM of active colonization of* A. brasilense *on sand*

Random dispersal of single cells or very small microcolonies characterized the population density of inoculated sand particles. Bacterial cell

size was smaller compared to cells which were grown in liquid medium (0.8-1.5 *m*m compared to 2-3 *m*m, respectively). Microcolonies were found in relatively small numbers (Fig. 1A), located mainly in the shallow cavities of quartz particles (unpublished data). Many bacterial cells were attached to the surface of the sand particles by fibrillar material, whereas, others lacked any visible connection or having few connections to the sand (Fig. 1B and Fig. 1B insert). This fibrillar material was found to be either single-stranded or multistranded, and it was located on any side of the bacterial cell (Figs. 2A, B). In addition to their connection to sand, bacteria in the microcolonies were also connected to each other by fibrillar material (Fig. 213, arrow). Connections were also found in aggregates of $agg^+$ cells grown in liquid culture (Figs. 3A, B) that were missing in $agg^-$ cultures (Figs. 3C, D). The possibility that the observed fibrils were bacterial flagella was examined and discarded. Flagella in strain Cd are much longer and have significantly smaller diameter (for comparison see Levanony and Bashan, 1989) that prevents their detection by SEM.

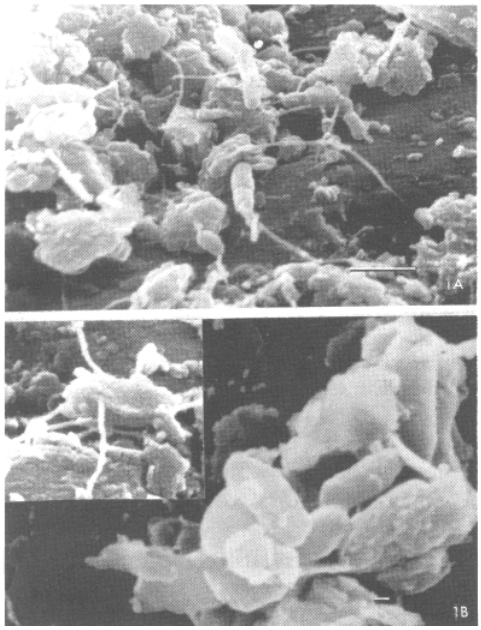


*Fig. 1.* Scanning electron micrographs of *A. brasilense* $agg^+$ cells colonizing sand. (**A**) random dispersal of single cells and small microcolonies in a shallow cavity on a sand particle. (**B**) bacterial microcolony lacking or having few fibrillar connections to the sand particle. *Insert*: attachment of cells to sand particle by fibrillar material. Bars represent 1 *m* (**A**) and 0.1 *m* (**B**).

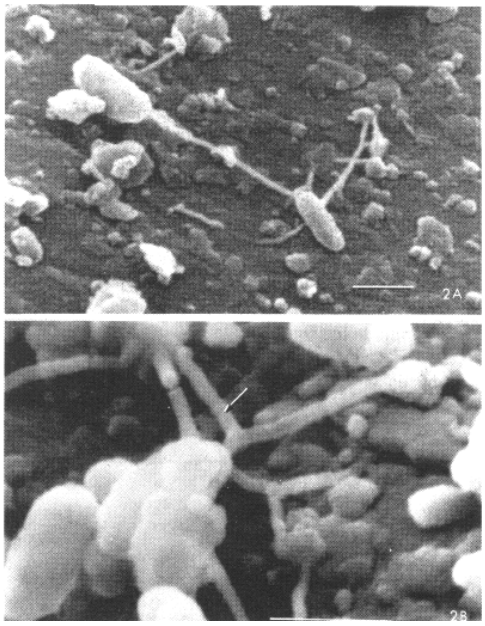


*Fig. 2.* Scanning electron micrographs of a single stranded (**A**) and multistranded (**B**) fibrillar material connecting *A. brasilense* $agg^+$ to sand. Arrow indicates fibrillar material connecting two bacterial cells. Bars represent 1 *m*.

### *Attachment of* A. brasilense *$agg^+$ and $agg^-$ to sand following washing*

Bacterial attachment to sand by both strains immediately after bacterial application was negligible (<0.001%). Population size of either $agg^+$ cells or agg- cells in sand was similar ($>10^7$ cfu $mL^{-1}$ after 48 h). However, percentage of attachment of the 2 strains to the sand differed significantly, with the $agg^-$ mutant showing lower attachment rates (Fig. 4). Slight rinsing of the sand (after sand colonization) decreased the adsorption of both strains, but there was a greater decrease in $agg^-$ cells. Increasing the washing time almost eliminated $agg^-$ cells from the sand, while significant number of $agg^+$ cells (approx. $10^6$ cfu $g^{-1}$ sand) remained attached to the sand. Despite a decrease in the total bacterial number of $agg^+$ cells caused by

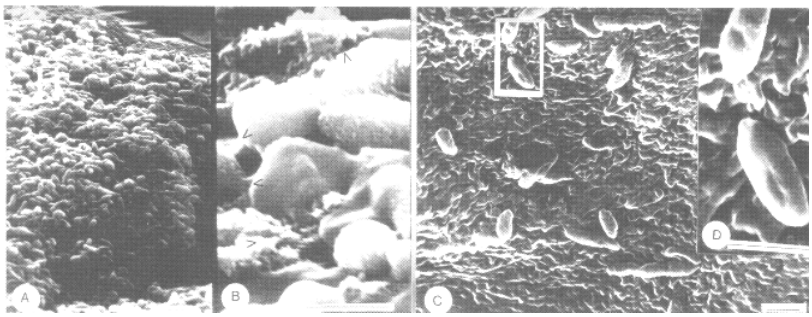

*Fig. 3.* Scanning electron micrographs of (**A**) aggregate of wild-type *A. brasilence* Cd; (**B**) Arrows indicate formation of fibrils and adhesive material between cells of *A. brasilense* agg in the aggregate (insertion in Fig. 3A shows location of Fig. 3B) and (**C**) lack of fibrils in agg$^-$ cells grown in nutrient broth liquid medium. (**D**) Enlargement of agg cells showing a smooth surface (insertion in Fig. 3C shows location of Fig. 3D). Bars represent 1 *m*

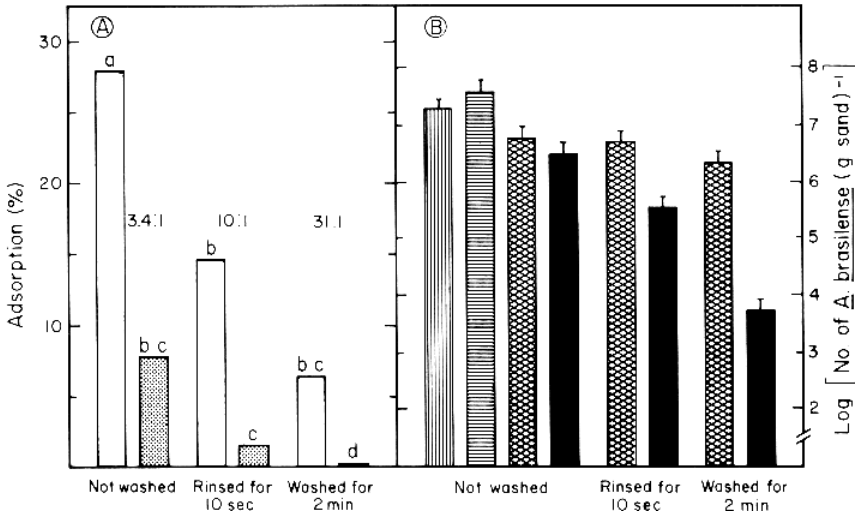


*Fig. 4.* **A**- Percentage of adsorption and **B**- number of cells of *A. brasilense* (agg$^+$) and *A. brasilense* (agg$^-$) in sand before and following washing treatments. □- adsorption of agg$^+$; ▨- adsorption of agg$^-$; ▩- number of cells of agg$^+$ adsorbed; ■- number of agg$^-$ cells adsorbed; total number of agg$^+$ cells (▥) and agg$^-$ cells (▤) in the sand mixture. Columns followed by a different letter differ significantly at $P \leq 0.05$. *Bars* represent SE. Numbers represent the adsorption ratio between agg$^+$ and agg$^-$ cells.

washing, the adsorption ratio between agg$^+$ and agg$^-$ strains increased with increasing the washing time about 10 times (Figs. 4A, B).

*Adsorption of* A. brasilense *agg$^+$ and agg$^-$ cells to sand after long incubation*

Both strains developed an equal bacterial populations after prolonged incubation time. However, longer incubations with bacteria in the sand increased percentage of adsorption of both strains. The ratio between agg$^+$ and agg$^-$ was lower compared to the standard conditions used in the rest of the study. Slight rinsing of the sand detached most bacteria from the sand particles, and similarly the adsorption ratio was lower compared under standard conditions (Table 2).

*Agitation effects on adsorption of* A. brasilense *agg$^+$ and agg$^-$ to sand*

Generally, agitation increased bacterial population in the sand and decreased their adsorption. Both strains developed large populations (> $10^8$ cfu g$^{-1}$ sand), and there was no significant difference between the strains. However, the

*Table 2.* Adsorption of *A. brasilense* (agg$^+$) and *A. brasilense* (agg$^-$) to sand after incubation for 10d

| Bacterial strain | Adsorption (%) | | Number of adsorbed bacteria | | Ratios of adsorption between agg$^+$ and agg$^-$ | | Total number of bacteria in sand |
|---|---|---|---|---|---|---|---|
| | Not washed | Rinsed | Not washed | Rinsed | Not washed | Rinsed | |
| agg$^+$ | 33.4aA [a] | 18.7aB | $1.8 \pm 0.3 \times 10^7$ | $9.9 \pm 0.2 \times 10^{6}$ [b] | 2.9:1 | 6:1 | $5.3 \pm 0.4 \times 10^7$ |
| agg$^-$ | 11.4bA | 3.16B | $6.9 \pm 0.4 \times 10^6$ | $1.9 \pm 0.5 \times 10^6$ | | | $6.1 \pm 0.6 \times 10^7$ |

[a]Numbers followed by different lower case letter in each vertical column or by capital letter in horizontal column differ significantly at $P \leq 0.05$.
[b]SE.

adsorption ratio (between agg$^+$ and agg$^-$) increased as a result of agitation. When strong agitation (60 sec) was applied to sand, nearly all agg$^-$ cells were desorbed from the sand particles (840 cells out of $10^8$ cfu g$^{-1}$ sand remained) (Figs. 5A, B).

*Effect of protease on adsorption* of A. brasilense *agg$^+$ and agg$^-$ to .sand*

Protease treatment of sand colonized by the two strains (separately) decreased adsorption of agg$^+$ and at the same time large amounts of unidentified yet proteinaceous substances) were released into the sand (Figs. 6A, B). Denaturated protease had no effect on adsorption. No protease effect was detected on agg$^-$ cells colonizing sand. The effect of protease on adsorption of these cells was not greater than rinsing alone (compare Figs. 4A, 6A). In addition, no measurable change in the protein content of the sand supernatant was detected. No effect on the viability of both strains by protease treatment could be detected (1.05 $OD_{540}$ = $10^9$ cfu mL$^{-1}$).

*Effect of EDTA added to sand on adsorption of* A. brasilense *agg$^+$ and agg$^-$*

Addition of NaEDTA prior to inoculation of the sand did not affect the growth in sand of either

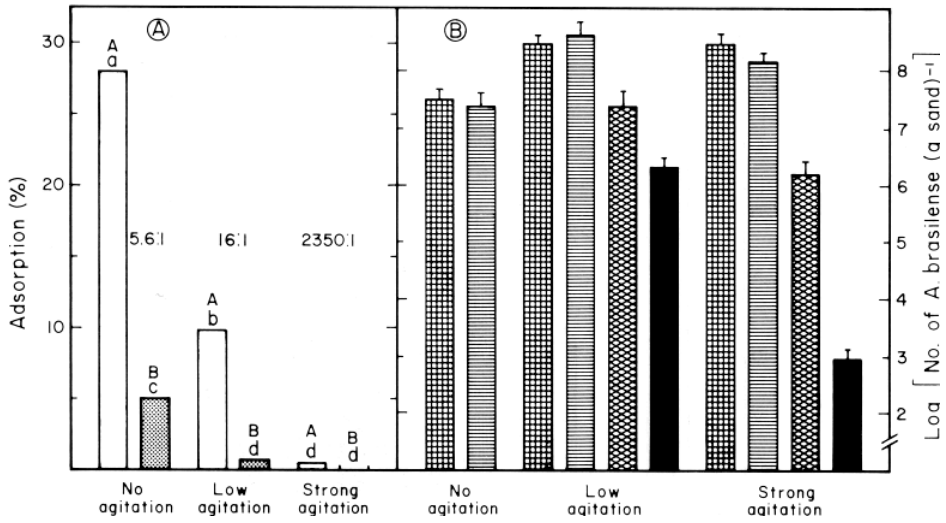


*Fig. 5.* **A**- Percentage of adsorption and **B**- number of cells of *A. brasilense* (agg$^+$) and *A. brasilense* (agg$^-$) in sand before and after agitation. □- adsorption of agg$^+$; ▨- adsorption of agg$^-$; ▩- number of cells of agg$^+$ adsorbed; ■- number of agg$^-$ cells adsorbed; total number of agg$^+$ cells (▦) and agg$^-$ cells (▤) in the sand mixture. Columns followed by a different lower case letter differ significantly at $P \leq 0.05$. Pairs of columns followed by a different capital letter differ significantly at $P \leq 0.05$ using t-test analysis. Bars repesent SE. Number represent the adsorption ratio between agg$^+$ and agg$^-$ cells.

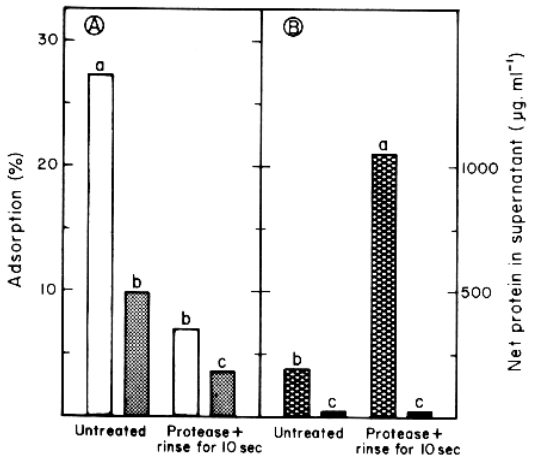


*Fig. 6.* **A**- Percentage of adsorption of *A. brasilense* (agg$^+$) and *A. brasilense* (agg$^-$) and **B**- changes in the protein content of sand supernatant after incubation of bacteria-sand mixture with 1% protease for 1 h. □- adsorption of agg$^+$; ▨- adsorption of agg$^-$; ▩- sand supernatant protein content by agg$^+$; ■- the same for agg$^-$. Columns followed by a different letter (in each sub-figure separately) differ significantly at $P \leq 0.05$.

strain. However, NaEDTA significantly decreased adsorption of agg$^+$ cells and had no effect on adsorption of agg$^-$ cells. The adsorption ratio between agg$^+$ and agg$^-$ was not significantly affected by EDTA treatment (1.2:1) (Fig. 7).

*The effect of clay addition on adsorption of* A. brasilense *agg$^-$ to sand*

Addition of montmorillonite had no effect on the size of the bacterial population that developed in the sand. However, it increased dramatically the adsorption of agg$^-$ cells to the "newly formed soil" particles similarly to adsorption of agg$^+$ under these conditions previously demonstrated (Bashan and Levanony, 1988a). The greater the amount of clay added (up to an upper limit of 5%), the higher agg$^-$ adsorption occurred (Fig. 8). Subjecting the "soil"-bacteria mixture to a protease treatment revealed no release of a detectable amount of protein into the "soil" supernatant (data not shown).

*Survival of* A. brasilense *agg$^+$ and agg$^-$ in sand*

Survival of the agg$^-$ strain was shorter than of agg$^+$. Few cells of agg$^-$ were detected only by liquid enrichment of the sand after 20 d, whereas, agg$^+$ cells were detected at that period of time by conventional plate count method, although in small numbers (Table 3).

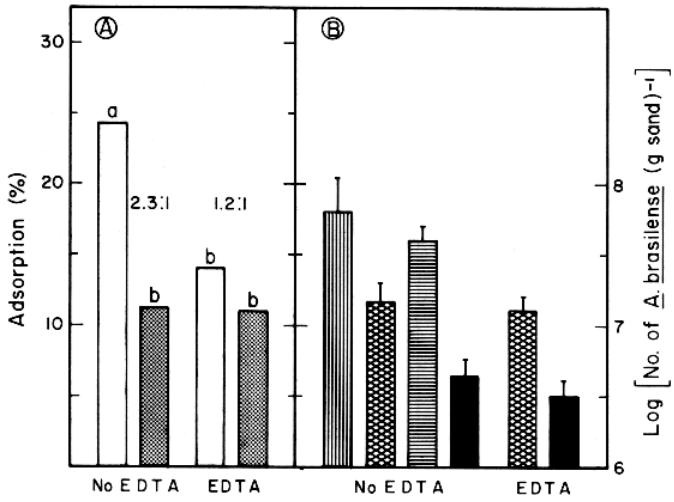


*Fig. 7.* **A**- Percentage of adsorption and **B**- number of cells of *A. brasilense* (agg$^+$) and *A. brasilense* (agg$^-$) in sand before and after NaEDTA treatment. □- adsorption of agg$^+$; ▨- adsorption of agg$^-$; ▩- number of cells of agg$^-$ adsorbed; ■- number of cells of agg$^-$ adsorbed; total number of agg$^+$ cells (▥) and agg$^-$ cells (▤) in the sand mixture. Columns followed by a different letter differ significantly at $P \leq 0.05$. Bars represent SE. Numbers represent the adsorption ratio between agg$^+$ and agg$^-$ cells.

*Table 3.* Survival of *A. brasilense* agg$^+$ and agg$^-$ in quartz sand

| Days after inoculation | No. of *A. brasilense* [cfu g$^{-1}$ sand] | | Detection of *A. brasilense* after liquid enrichment | |
|---|---|---|---|---|
| | agg$^+$ | agg$^-$ | agg$^+$ | agg$^-$ |
| 1 | 1 x lO$^8$a[a] | 1 x 10$^8$a | NT | NT |
| 10 | 4.6 ± 0.4 x 10$^4$b | 3.2 ± 0.5 x 10$^3$c | NT | NT |
| 20 | 8.2 ± 0.6 x 10$^1$ d | 0 | NT | + |
| 25 | 0 | 0 | + | - |

NT-not tested.
5 x 10$^8$ cfu *A. brasilense* in the logarithmic phase of growth were applied to 5 g sand.
[a]Numbers followed by a different letter differ significantly at $P \leq 0.05$.

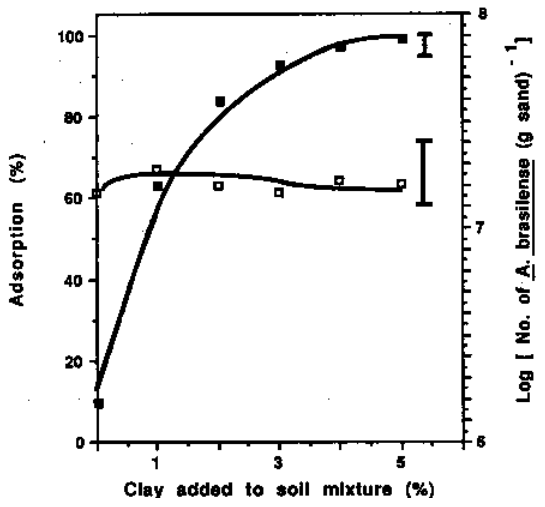


*Fig. 8* Adsorption of *A. brasilense* (agg) to sand supplemented with clay (montmorillonite). ■ - Percentage of adsorption; □- number of bacteria developed in the sand. Bars represent the SE of the lines.

## Discussion

Azospirillum is inoculated into the soil for plant root colonization and enhanced plant growth and yield (Bashan, 1986; Michiels *et al.*, 1989; Smith *et al.*, 1984). However, when the bacterial cells are applied to an environmental habitat such as quartz sand, which holds them very loosely, they are readily detached by any water stream. Therefore, the bacterial cell should produce anchoring substance(s) in order to prevent this undesired washing into deeper soil layers *i.e.*, below the root system.

*A. brasilense* Cd is known for forming large bacterial aggregates as are nearly most Azospirillum strains, both in liquid medium (Levanony and Bashan, 1989; Madi *et al.*, 1988) and on root surfaces (Bashan and Levanony, 1990). Recently, we have shown that *A. brasilense* Cd is actively attached to sand particles by a network of proteinaceous bridges (Bashan and Levanony, 1988b). Therefore, the focus of this study has been on the role of these bacterial fibrils in sand, in order to establish if they aid bacterial persistence in this environment.

Desorption of soil bacteria, as well as rhizosphere bacteria such as *A. brasilense* Cd, from soil particles by external mechanical forces such as washing and agitation is difficult. Bacterial cells are strongly and permanently adsorbed to the soil particles (Bashan and Levanony, 1988a; Hattori, 1988; Marshall, 1980). However, attachment of *A. brasilense* Cd to quartz sand particles is relatively weak. This study presents scanning electron microscope evidence, combined with quantitative analysis of these fibrils, demonstrating that single and multistranded fibrillar material are present and provide washing resistance for the cells. Such phenomenon did not occur when a mutant incapable of producing these fibrils was inoculated into the sand. Furthermore, although agitation caused partial desorption of agg$^+$ cells, it had even a stronger effect on agg$^-$, eliminating nearly all the bacterial cells from the sand. Therefore, it can be further concluded that this network provides resistance against external physical forces applied to sand.

Fibrillar connections between bacterial cells in bacterial aggregates and between bacterial cells and soil particles can vary in their chemical composition. Two species of rhizobia were

bound to silt particles by fibrillar polysaccharide substances) (Fehrmann and Weaver, 1978). Both in liquid culture and in sand, protein connections were found in *A. brasilense* Cd (Bashan and Levanony, 1988b; Madi *et al.,* 1988). The present study provides further evidence that proteinaceous compounds) may be involve in these bindings; protease treatment had a marked effect on desorption of only the aggregating wild-type strain ($agg^+$).

Clays and organic matter in the soil play the major role in bacterial adsorption to the soil particles (Marshall, 1980) and in soil aggregation (Lynch and Bragg, 1985). Bacteria attached mainly to the colloidal fraction of the soil. Montmorillonite in particular is known to adsorb large quantities of bacterial cells (Marshall, 1968). Addition of clay and organic matter to sand resulted in adsorption of *A. brasilense* Cd to sand in a fashion similar to that of soil (Bashan and Levanony, 1988a). This study gives further evidence to the role of clay-bacteria adsorption features of *A. brasilense* Cd. When clay was added to sand in small quantities, the non-aggregating mutant ($agg^-$) strongly adsorbed to the new formed "soil". Since no production of external fibrils was detected, it was concluded that this type of adsorption is probably of charge-charge attraction, which is a common adsorption /desorption mechanism in most soils (Daniels, 1980). Thus, it can be suggested that in pure sand, the fibrillar network has a role in *A. brasilense* Cd attachement.

Survival of *A. brasilense* Cd in soils is short in the absence of plants, decreasing to a minimal population size after less than 20 d (Albrecht *et al.,* 1983; Bashan and Levanony, 1987; 1988a; Bashan *et al.,* 1987; Smith *et al.,* 1984). Despite this, the present study gives evidence that the ability to produce fibrillar connections is advantageous and given prolonged survival period to the strain capable of this production. Probably, it reflects an ecological adaptation of the wild-type strain to unfavorable environments.

In conclusion, our study suggests that fibrillar material plays an important role in attachment of *A. brasilense* Cd to sand particles. The importance of the fibrillar network is mainly for providing resistance against external physical forces applied to the sand, thus, increasing survival. Nevertheless, they are not essential for multiplication of the bacteria in the sand. The applicative relevance of this attachment to sand and its impact on root colonization is currently under study.

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