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Localization of Specific Antigens of *Azospirillum brasilense* Cd in Its Exopolysaccharide by Immuno-Gold Staining

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Abstract. Elicitation of specific antibodies towards *Azospirillum brasilense* Cd was performed by injecting whole, living cells. The antigens which caused this specific response were detected by the immuno-gold technique and found to be located in the exopolysaccharide layer of the bacterial cells.

Inoculation of grasses with *Azospirillum* strains is being evaluated worldwide owing to their potential contribution to plant productivity [20]. One of the major problems in *Azospirillum* research is the identification and enumeration of species of this genus on plant roots. The most common identification methods are based on semi-selective media and bacterial enrichments [2, 19]. However, these methods are not specific enough and can be considered, in general, unreliable. Despite a decade of research, immuno-detection methods are not common, and very few studies have been published describing the antigenic characteristics of species in this genus [8, 10, 11, 22]. Nearly all studies employed polyclonal antibodies, but only a few [15, 17, 22] demonstrated antibodies specific to *Azospirillum* strains. The location of the antigens of *Azospirillum* that elicit antibodies has not been elucidated.

The aim of the present study was to locate the sites of the antigens of *A. brasilense* Cd, which elicited highly specific antibodies.

Materials and Methods

Bacteria. *Azospirillum brasilense* Cd (ATCC 29710) was used as the standard strain. Other bacteria used were *A. brasilense* Sp-7 (ATCC 29145); site-directed nif$^-$ mutant of strain Cd (29710 nif (205 B-1)-10b)); auxin-overproducing mutants of *A. brasilense*, FT-326 and FT-400 [12]; *A. brasilense* No. 5 isolated from roots of *Chloris gayaner*, No. 6 isolated from the rhizosphere of the same plant, and No. 11 isolated from the rhizosphere soil of *Urochloa mosambicensis*. All these isolates were originally isolated from northeastern Brazil. Biotin-requiring *A. brasilense* No. 68, isolated from roots of an unidentified grass from sea water, affected soil south of Rio de Janeiro (B. Reinhold, personal communication); the *A. brasilense*-like strain 82012 [1] and four unidentified *Azospirillum* strains #1, #2, #3, #4; *A. halopraeference* No. 4 (type strain, B. Reinhold, personal communication); rhizosphere bacteria strain 84072 isolated from wild relatives of wheat growing in Israel; other rhizosphere bacteria, strains 1013, 1015, 1019, 1020, and 1023, and four unidentified rhizosphere strains, #5, #6, #7, and #8, isolated from roots of cultivated wheat; the saprophytic strain 82005 from our laboratory collection (Y. Bashan and H. Levanony, unpublished data); and the pepper leaf pathogen *Xanthomonas campestris* pv. *vesicatoria* (ATCC 11633).

Culture conditions and bacterial counts. *Azospirillum* strains and *Xanthomonas campestris* pv. *vesicatoria* were cultured in nutrient broth (Difco) in a rotary shaker (200 rpm) at 30 ± 2°C for 24–48 h to a final concentration of 10^9 colony-forming units (cfu)/ml. Other rhizosphere bacteria were similarly cultured at 22 ± 2°C. *Azospirillum brasilense* Cd for gold labeling was grown in still cultures, without shaking, in a nitrogen-free synthetic medium for *Azospirillum* [19]. Bacterial counts were performed by the plate-count method on nutrient agar (Difco) plates, 48 h after plate inoculation [2]. Indirect enzyme-like immunosorbent assay (ELISA) was performed as previously described in detail [15].

Antisera production and IgG purification. Whole cells of *A. brasilense* Cd were used to elicit antibodies. Cells were harvested from the liquid culture by centrifugation at 12,000 *g* for 10 min at 4 ± 1°C and washed three times in sterile potassium phosphate-buffered saline (PBS), pH 7.2, and their numbers were adjusted to 10^9 cfu/ml (1.05 A_{540} units). The bacterial suspension was emulsified with an equal volume of complete Freund adjuvant. Antibodies were elicited in New Zealand white rabbits by immunization with multiple intradermal injections with 1 ml of bacterial emulsion at four 1-week intervals, and a booster was given after an additional 2 weeks. Bleeding via cardiac puncture was started in week 2 post immunization and continued for 3 months at 10-day intervals. Antisera from individual bleeding were stored at −20°C. Before use, the antisera were tested for their ability to induce agglutination, by using 10^8 cfu of the antigen suspended in 200 μl of PBS in microtiter plates. The antisera used in this work had an initial titer of 1 : 512 by this method.

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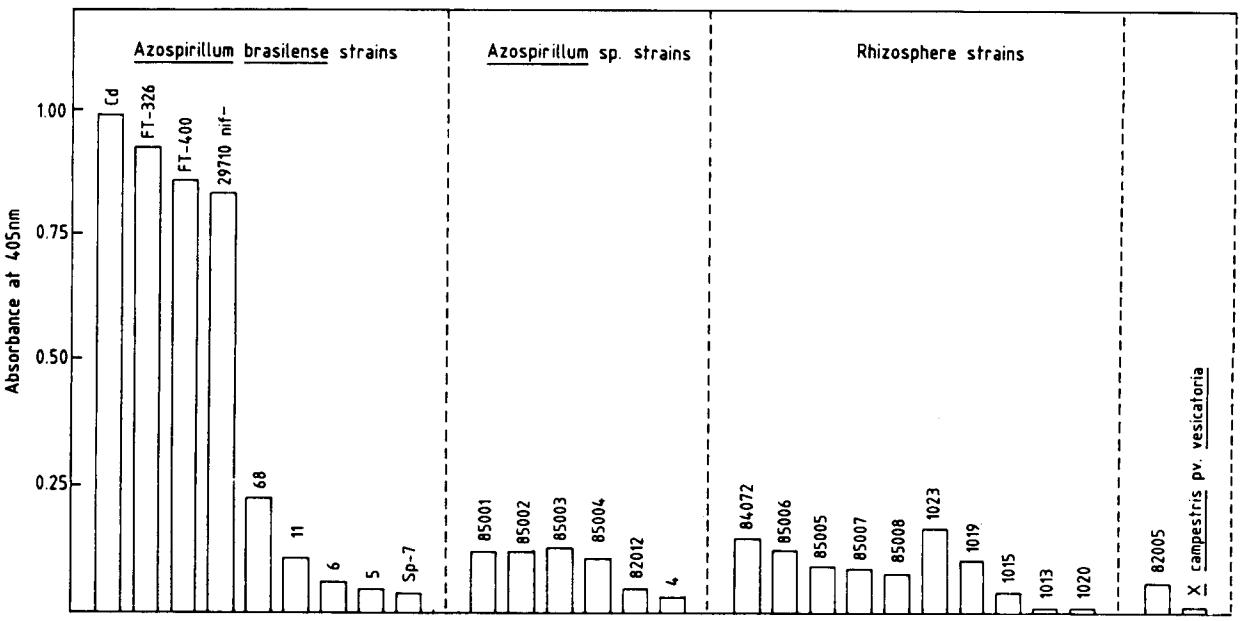

Fig. 1. Specificity and cross-reactions of anti-*A. brasilense* Cd towards various bacterial isolates analyzed by indirect ELISA. All isolates were tested at 10^8 cfu/ml.

To minimize nonspecific interactions, gamma-globulin was purified as follows. Samples (1 ml) of antisera were diluted tenfold with distilled water, and 10 ml of a saturated solution of ammonium sulfate (pH 5.5) was then added to each diluted sample. After 1 hour at room temperature, the formed precipitate was collected by centrifugation at 12,000 *g* for 15 min, dissolved in 2 ml of half-strength PBS, and dialyzed overnight at 4 ± 1°C against three changes of 500 ml of half-strength PBS–0.02% sodium azide. The IgG was further purified on a column (1 × 8 cm) of DEAE–cellulose (DE-23, Whatman), which was previously equilibrated with half-strength PBS–0.02% sodium azide. The unadsorbed fraction was adjusted to 1 mg/ml (E_{280} = 1.4) and stored frozen at −20°C in 1-ml microtubes [15]. After purification, these polyclonal antibodies showed high specificity towards *A. brasilense* Cd with negligible cross-reaction to the other rhizosphere bacteria listed above as well as towards other *Azospirillum* species (Fig. 1 and Ref. [15]).

Antigen detection by immuno-gold technique. The entire procedure was carried out at room temperature. All buffers and other solutions were filtered through 0.45 μ filters (FP 030/2, Schleicher and Schuell, USA). The entire procedure described below was performed by passing the grids from one drop of solution to the next drop, which were placed on a parafilm layer. Nickel grids supported with a film of Parlodion (USA) and carbon coated were immersed for 2 min in 0.01% poly-L-lysine. Bacterial cells (10^8–10^9 cfu/ml) were adsorbed to the grids for 10 min. Bacteria were taken directly from the liquid culture in order to preserve their original shape and flagella; *A. brasilense* Cd cells were fixed for 10 min in 2% glutaraldehyde (Polysciences, Warrington, Pennsylvania) in 0.1 *M* cacodylate buffer, pH 7.2. Each grid was rinsed in eight drops of double-distilled water. Nonspecific binding was blocked for 10 min with 1% egg albumin (grade V, Sigma Chemical, St. Louis, Missouri) in PBS, pH 7.2, supplemented with 0.05% Tween-20 (Sigma) and 20 m*M* NaN_3. The grids were transferred to specific anti-*A. brasilense* Cd primary antibody, diluted 1:1000 in PBS supplemented with 0.05% Tween-20 and 20 m*M* NaN_3, for 90 min at room temperature. The grids were then rinsed in the above buffer, and blocking was repeated with 1% egg albumin for 10 min. The grids were incubated with the secondary antibody, goat anti-rabbit immunoglobulin conjugated to colloidal gold (AuroProbe-EM, GAR-G15, Janssen, Belgium) diluted 1:10 in Tris-buffered saline (TBS) (20 m*M* Tris HCl, 0.15 *M* NaCl, pH 7.4) for 30 min. The grids were rinsed first in TBS (six washings), then several times in double-distilled water (5 min each washing) and examined under TEM. Various solution concentrations, buffers, and incubation periods were tested, and the above procedure was found to be optimal for observing those bacteria.

Experimental design. All experiments were randomly designed in triplicate, with two to six wells in a microtiter plate as a single replicate. Experiments were repeated two to four times each. Each ELISA plate contained controls including a thawed culture of *A. brasilense* Cd of a known dilution. This culture was used throughout the study for comparing different performances of the microtiter plates. Controls used in this study were: preimmune sera; wells with a conjugate or substrate but without antibodies or antigens; unlabeled bacteria; and gold labeling of bacteria without primary antibodies.

Results and Discussion

The use of antibodies for the specific identification of beneficial bacteria associated with plant roots has gained popularity during the last decade. Of the many different immunological methods, the enzyme-linked immunosorbent assay (ELISA) be-

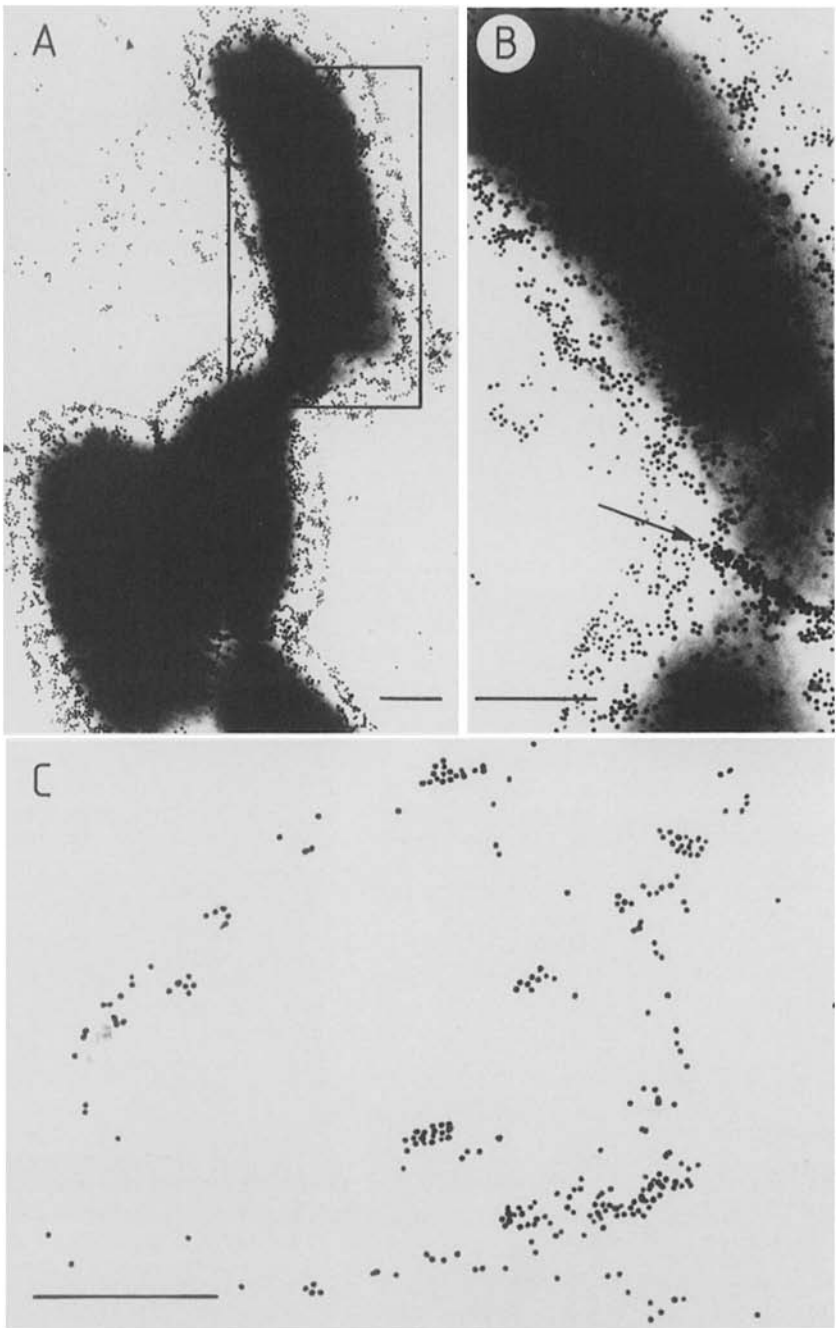

Fig. 2. Specific labeling of *A. brasilense* Cd EPS by colloidal gold particles. (A) four labeled bacterial cells; (B) insertion in Fig. 2a showing a "cloud" of gold particles labeling around the cell, note condensed labeling at the junction between two cells (arrow); (C) labeling of EPS after spontaneous removal of the bacterium cell revealing the image of the bacterium. Bars represent 0.5 μm.

came the one most commonly used [5–7, 14–18, 23]. Result reliability, in these methods, is totally dependent on antibody specificity. The usual procedure of producing antibodies for detection of bacteria is by injection of whole, viable or dead, bacterial cells, and then minimal purification of the antisera. The quality of antibodies thus produced depends on the titer and on antibody specificity. However, since different specific antigens are present on the bacteria, they may initiate polyclonal specific antibodies.

The few immunological studies on *Azospirillum* yielded two different observations: (i) strain specificity at high levels with no cross-reactions [15, 17, 22]; (ii) some or extensive cross-reactions between strains [8, 10]. The results presented herein support the specificity approach. Testing of 21 different strains of rhizosphere bacteria, including 12 strains

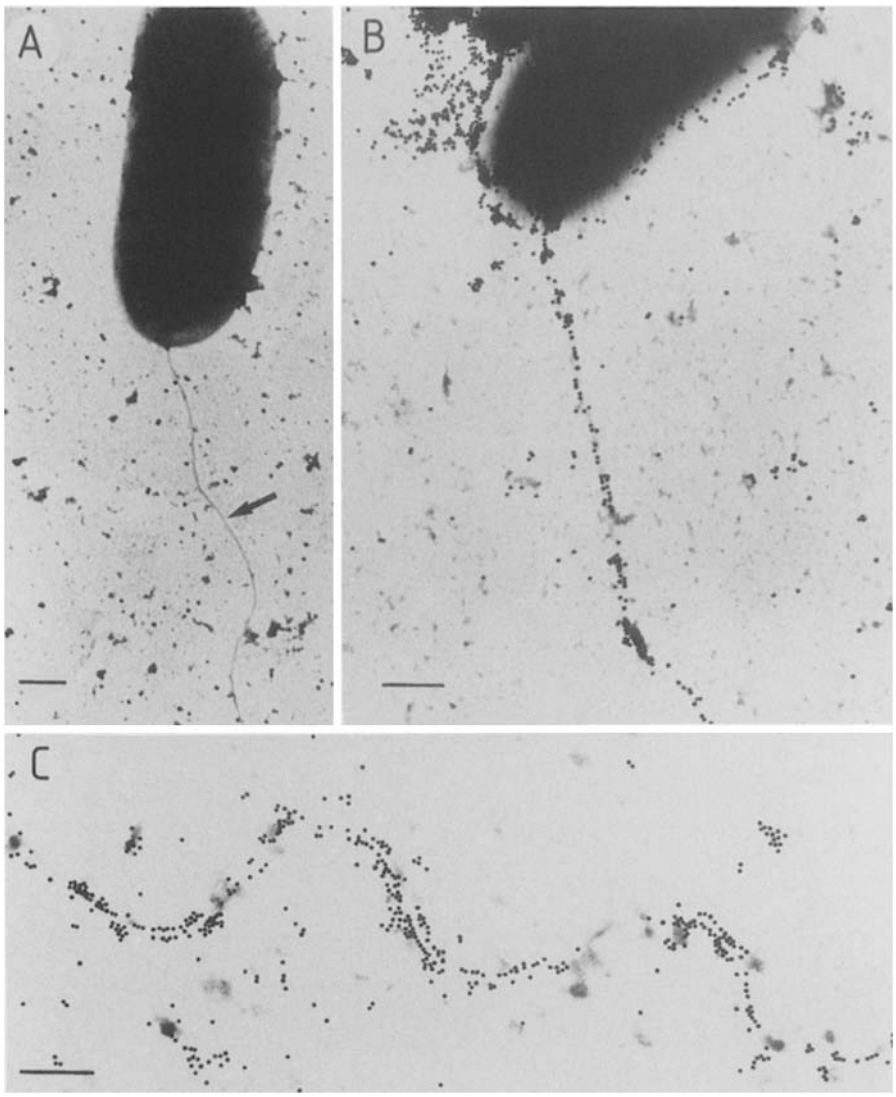

Fig. 3. Specific labeling of *A. brasilense* Cd antigens around the bacterium flagellum. (A) gold labeling without primary antibodies (control); arrow shows the flagellum; (B) flagellum gold labeling; (C) gold labeling of the entire flagellum. Bars represent 0.5 μm.

of *A. brasilense* and one phytopathogenic bacterium, revealed that cross-reaction with anti-*A. brasilense* Cd is negligible. Only very high bacterial concentration, 10^{10}–10^{11} cfu/ml, caused some ELISA readings. High binding values were obtained for *A. brasilense* Cd and its mutants (FT-326, FT-400, and 29710 nif$^-$) (Fig. 1).

The use of immunogold cytochemistry for ultrastructural detection of cell substances has recently increased, mainly for animal tissue. It is now considered one of the most precise techniques for detecting and identifying substances at the cellular level [9, 21]. However, this technique is rarely used in phytobacteriology [23]. Recently, it was used to show that *A. brasilense* Cd colonized root intercellular spaces [3] and to detect *Rhizobium loti* bacteriodes in *Lotus* roots [13].

In this study we demonstrate that the specific antigens of *A. brasilense* Cd are visibly located in the exopolysaccharide (EPS) layers encapsulating each bacterial cell (Fig. 2a,b). Preliminary experiments have shown that these specific antibodies precipitated *A. brasilense* Cd EPS (unpublished data). Gold labeling was dense, and the background composed from random nonspecific binding of gold particles was very low. When bacterial cells were spontaneously released from the TEM grid but their EPS layer remained stuck to the surface, the specific antibodies recognized the remaining EPS and bound to it. Later application of the gold-labeling technique revealed the shape of bacteria previously located in this site (Fig. 2c). In addition, some of the specific antibodies were bound to the outer surface of the bacterium's single flagellum (Fig. 3b,c). *Azo-*

spirillum cells are known to produce a single polar flagellum when grown in liquid culture [11]. Similar specific labeling of bacterial pili and flagella was previously shown by the immuno-gold technique in *Bacteroides nodosus*, causing sheep footrot disease [4], and in *A. brasilense* by the immunoperoxidase stain technique [11].

In conclusion, this study suggests that the specific polyclonal antibodies elicited by injecting whole *A. brasilense* Cd cells are located in the exopolysaccharide layer of the bacterium cell.

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