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 gayanae, 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 10⁸ 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 10⁹ cfu/ml (1.05 A₅₄₀ units). 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].
To minimize nonspecific interactions, gamma-globulin Cd was purified as follows. Samples (1 ml) of antisera were diluted ten-fold 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 \( \times \) 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 x 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 (E280 = 1.4) and stored frozen at -20°C in 1-ml microtubes [15]. After purification, these polyclonal antibodies showed high specificity towards \( A. \) brasilense \( \text{Cd} \) with negligible cross-reaction to the other rhizosphere bacteria listed above as well as towards other \( A. \) brasilense species (Fig. 1 and Ref. [15]).

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 \( \text{Cd} \) 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-
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...
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<sup>-</sup>) (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-
spiritum 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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