

## Production and Characterization of Strain-Specific Monoclonal Antibodies against Outer Membrane Components of *Azospirillum brasilense* Sp245

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### ABSTRACT

Several hybridoma cell lines producing murine monoclonal antibodies (MAbs) directed against outer membrane components of the Gram-negative rhizosphere bacterium *Azospirillum brasilense* Sp245 have been established and characterized. Whole bacterial cells were used as immunogens. Among the clones obtained, 14 hybridoma cell lines were selected for further characterization. Eight MAbs were strain-specific and 6 MAbs showed cross-reactivity with a closely related strain *Azospirillum brasilense* Sp246. According to the biochemical characterization of the antigenic determinants, MAbs were classified into four groups. The corresponding antigens were lipopolysaccharides (class 1) and an outer membrane protein (class 4), which is common to *Azospirillum brasilense* Sp245 and *Azospirillum brasilense* Sp246 as well as two outer membrane proteins (class 2 and class 3) that are characteristic for *Azospirillum brasilense* Sp245. The number of antigens per cell varied from 4000 (class 1) to 100 (class 4). In each class high affinity MAbs were identified, which made a sensitive direct quantification of *Azospirillum brasilense* Sp245 possible.

### INTRODUCTION

BACTERIA OF THE GENUS *Azospirillum* belong to the group of  $\alpha$ -proteobacteria.<sup>(1)</sup> They have been isolated mainly from the rhizosphere of a variety of tropical and subtropical plants.<sup>(2)</sup> Their potential to fix atmospheric nitrogen<sup>(3)</sup> and to produce different phytohormones<sup>(4)</sup> is of great interest to enhance crop growth using bacterial inoculants. The most marked effects of *Azospirillum* inoculation are morphological changes induced in the root system. Many studies have demonstrated positive effects of inoculation on root parameters, such as root length,<sup>(5)</sup> number of lateral roots,<sup>(6)</sup> increase in the number of root hairs,<sup>(7)</sup> and enhanced cell division in the root meristem.<sup>(8)</sup> Due to these properties, bacteria of the genus *Azospirillum* are considered as potential plant growth-promoting rhizobacteria (PGPR). Effects of *Azospirillum* inoculation on the total yield of field grown crops generally range from 10 to 30% increase.<sup>(9,10)</sup> These moderate yield increases, attributed to inoculation with *Azospirillum*, are sufficient to be commercially important, if

they could be obtained consistently. In contrast to the very specific *Rhizobium*-legume symbiosis, *Azospirillum* strains interact with a variety of different crop plants.<sup>(11)</sup> *Azospirillum* colonizes the roots mostly externally.<sup>(12)</sup> They form small aggregates on the root surface and are embedded in the mucigel layer.<sup>(13)</sup> However, due to the possibility of displacement by other bacterial species, the number of *Azospirillum* in the rhizosphere is mostly variable<sup>(14)</sup> and depends on the bacterial strain.<sup>(15)</sup> It has been demonstrated for the strain *Azospirillum brasilense* Sp245 that it is able to colonize the root interior.<sup>(16)</sup> It was frequently found in intracellular spaces of the cortex and the vascular system of wheat.<sup>(17)</sup> Although most rhizosphere bacteria are not able to penetrate into the root interior, *Azospirillum brasilense* Sp245 can colonize the root interior very effectively.

Experiments describing the colonization of crop roots by *Azospirillum brasilense* Sp245 were performed in axenic systems.<sup>(18)</sup> To quantify and localize *Azospirillum brasilense* Sp245 in nonsterile soil systems, strain-specific monoclonal an-

tibodies (MAbs) for *Azospirillum brasilense* Sp245 were produced.

This paper describes the production and characterization of the strain-specific monoclonal antibodies for *Azospirillum brasilense* Sp245. The supernatants of hybridoma cells were screened by enzyme-linked immunosorbent assay (ELISA) on whole cells to select MAbs that detect cell surface antigens. The specificity of the selected MAbs was tested against different strains of *Azospirillum brasilense*, other *Azospirillum* species, and a panel of different bacteria representing the bacterial microflora of the rhizosphere. Antigens of the selected MAbs were characterized biochemically by one-dimensional (1D) and two-dimensional (2D) gel electrophoresis of the outer membrane components of *Azospirillum brasilense* Sp245 and western blotting. The number of antigens per cell was determined by immunogold-labeling and transmission electron microscopy (TEM) of embedded and ultrathin sectioned bacteria. The sensitivity of the MAbs was determined by a quantitative immunoassay based on chemoluminescence.<sup>(18)</sup> Information about differences in the affinity of the MAbs binding to the same antigen was investigated in a displacement experiment.

## MATERIALS AND METHODS

### Bacterial strain and cultivation

The bacterial strain *Azospirillum brasilense* sp245 has been isolated from an inner root tissue of wheat<sup>(16)</sup> and characterized as *Azospirillum brasilense* by physiological properties and its 23 S rRNA partial sequence.<sup>(11)</sup> All other bacterial strains were obtained from the German Collection of Microorganisms (DSMZ, Braunschweig, Germany), the American Type Culture Collection (ATCC, Rockville, MD), and the Laboratorium voor Microbiologie (LMG, Gent, Belgium). All strains were grown overnight in Luria Broth medium (LB).

### Mab production

Female mice BALB/c, 6–8 weeks old were immunized on day 0 by intraperitoneal (ip) injection with  $10^8$  living bacteria (in 100  $\mu$ l 10 mM Tris-HCl, pH 8). Three similar injections were done on days 21, 42, and 56. Five days after the final boost X63Ag8 cells were fused, according to Galfré and Milstein,<sup>(19)</sup> with spleen lymphocytes from the immunized mouse. Culture supernatants were screened 14 days after fusion for their content of antibodies against *Azospirillum brasilense* Sp245. Cells from positive wells were recloned several times and culture supernatants were tested against a panel of bacteria. Hybridomas of interest were cloned. The isotype and light chain type of the MAbs was determined by ELISA. The MAbs were purified by separating from other serum proteins with protein A treatment (Bio-Rad, Munich, Germany).<sup>(20)</sup>

### Immunoassays

All immunoassays were performed in 96-well PVC microtiter plates (Flow, Meckenheim, Germany) with an antirabbit-peroxidase-coupled secondary antibody (Amersham, Braunschweig, Germany) and ABTS (Boehringer, Mannheim, Germany) as substrate. The quantitative immunoassay was per-

formed in 96-well white-colored PE microtiter plates (Merlin, Hamburg, Germany) with an antirabbit-peroxidase-coupled secondary antibody and luminol (Amersham) as substrate.<sup>(18)</sup>

### Affinity displacement

A binding experiment (displacement study) was used to compare the affinity of MAbs, which bind to the same antigen. A nonlabeled (cold) MAb competes with a peroxidase-labeled (hot) MAb for the antigen binding site. The hot MAbs were coupled with peroxidase (Sigma, Munich, Germany).<sup>(20)</sup> Then  $10^7$  cells/well of an overnight culture of *Azospirillum brasilense* Sp245 were sorbed to a 96-well white-colored PE microtiter plate (Merlin).<sup>(18)</sup> The microtiter plates were incubated, blocked, treated with the peroxidase-coupled MAb (50 ng MAb/well), and washed.<sup>(18)</sup> Afterward the cold MAb was added in different amounts. The microtiter plates were incubated for 90 min at room temperature and washed five times. For the detection of the hot MAb that was still bound after displacement, luminol was used.<sup>(24)</sup>

### Isolation of outer membrane proteins

Outer membrane components were isolated by the method described by Bachhawat and Ghosh<sup>(21)</sup> with some modifications. About  $4 \times 10^9$  stationary phase cells grown in LB medium were washed twice with 10 mM Tris-HCl, pH 8. The washed cells were sonicated for 6 min at 80 W. The unbroken cells were removed by centrifugation for 10 min at 5000g. The supernatant was centrifuged at 600,000g for 30 min at 5°C. The pellet, consisting of the crude envelope fraction, was resuspended in 8 ml of a 10 mM Tris-HCl buffer, pH 8, containing 0.5% *N*-lauryl-sarcosine. After incubation for 30 min at 28°C, the suspension was centrifuged at 60,000g for 30 min, at 5°C. The pellet containing the outer membrane components was resuspended in 100  $\mu$ l of 10 mM Tris-HCl buffer, pH 8. It was shown by Bachhawat and Ghosh that this procedure yields an *Azospirillum brasilense* outer membrane preparation that is not significantly contaminated with cytoplasmatic or inner membrane proteins (NADH oxidase and succinate dehydrogenase activity was measured). Subsequently, proteins were isolated by phenol extraction.<sup>(22)</sup>

### Lipopolysaccharide extraction

Outer membranes were prepared as described above using  $1 \times 10^8$  stationary phase cells (grown at 33°C in LB-medium), but the final phenol extraction was omitted. The lipopolysaccharide fraction was obtained by proteinase K treatment (25 mg/ml) at 37°C overnight.<sup>(23)</sup>

### 1D and 2D gel electrophoresis

1D gels were done as SDS-polyacrylamide pore gradient gels (10–22%) with 4% stacking gels on a horizontal apparatus prepared according to Laemmli.<sup>(24)</sup> The dimensions of the gels were 30  $\times$  20  $\times$  0.02 cm. Tris-HCl (0.375 M) pH 8.8 was used as gel buffer. 2D gels were carried out according to O'Farrell<sup>(25)</sup> as described by De Mot and Vanderleyden.<sup>(22)</sup> The gels were transferred for western blotting or stained with Coomassie brilliant blue or silver.<sup>(21)</sup>



TABLE 2: RESULTS OF IMMUNOGLOBULIN CLASS DETERMINATION OF MABS MIPE 3-1.1 - MIPE 1-106 BY ELISA

	Mipe 3-1.1	Mipe 3-1.2	Mipe 3-1.3	Mipe 3-1.4	Mipe 3-1.6	Mipe 3-1.7	Mipe 3-1.8	Mipe 3-1.10	Mipe 3-1.11	Mipe 3-1.12	Mipe 1-103	Mipe 1-104	Mipe 1-105	Mipe 1-106
Ig M	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ig G <sub>1</sub>	-	+	+	+	+	-	-	-	-	-	+	-	-	-
Ig G <sub>2a</sub>	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Ig G <sub>2b</sub>	-	-	-	-	-	-	-	-	-	-	-	+	+	-
Ig G <sub>3</sub>	+	-	-	-	-	-	+	+	+	+	-	-	-	-
K	-	+	+	+	+	+	+	+	+	+	+	+	+	-

onto nitrocellulose membranes and incubated with the MABs. Figure 1a shows a 2D gel of outer membrane proteins of *Azospirillum brasilense* Sp245. The corresponding western blot with the MABs is shown in Figure 1b-e. Figure 2a shows a 1D

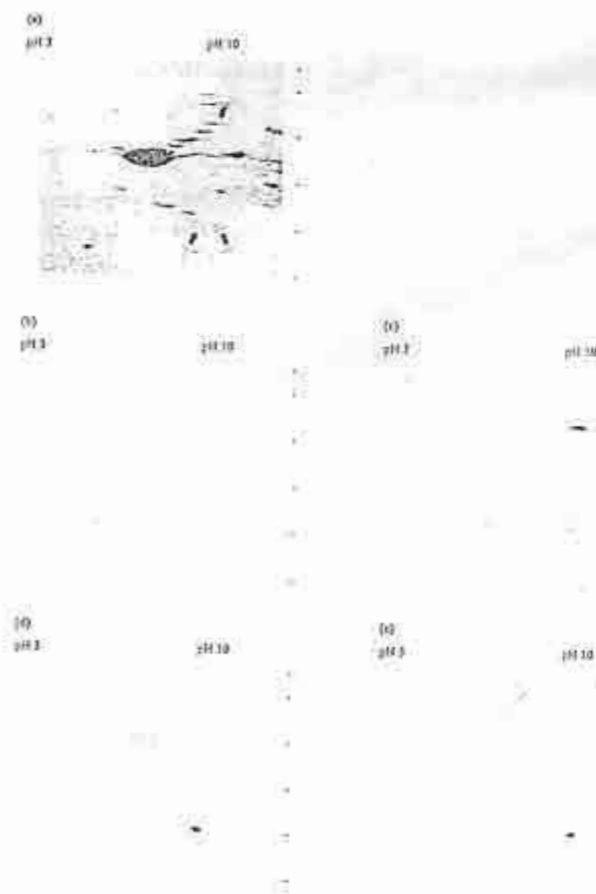


FIG. 1. Biochemical characterization of the antigenic epitopes for the *Azospirillum brasilense* Sp245-specific MABs. (a) The proteins were separated in the first dimension by IEF and in the second dimension by SDS-PAGE. In each gel the pH gradient extends from right (basic) to left (acidic). The positions of marker proteins are indicated by arrows, which correspond to, in descending order, an  $M_r \times 10^3$  of 97.4, 66.2, 42.7, 31.0, 21.5, and 14.4. The gel was stained with Coomassie brilliant blue. (b)-(e) Western blot of a 2D-PAGE separated outer membrane protein preparation of *Azospirillum brasilense* Sp245 (a) and immunoanalysis with MABs of classes 1-4 (Table 3). The corresponding proteins are marked in (a) with arrows.

gel of a lipopolysaccharide extract of *Azospirillum brasilense* Sp245. The corresponding western blot with the MABs is shown in Figure 2b-e. According to the blot results the antibodies were classified in 4 groups (Table 3). MABs of class 1 react with LPS, which is common to *Azospirillum brasilense* Sp245 and *Azospirillum brasilense* Sp246 (data not shown). MABs of class 2 bind to a 80-kDa outer membrane protein with an isoelectric point of 8.0, which is specific for *Azospirillum brasilense* Sp245. MABs of class 3 react with a 30-kDa outer membrane protein with an isoelectric point of 7.0, which is specific for *Azospirillum brasilense* Sp245. Class 4 MABs bind to a 30-kDa outer membrane protein with an isoelectric point of 8.0, which is common to *Azospirillum brasilense* Sp245 and *Azospirillum brasilense* Sp246.

#### Quantification of the antigenic determinants

To quantify the antigenic determinants of the MABs, an overnight culture of *Azospirillum brasilense* Sp245 was embedded in resin, ultrathin sections were cut, and treated with representative MABs of all four classes, which were coupled with gold particles. Figure 3a-d shows TEM picture of the bacteria with gold-coupled antibodies of classes 1-4. It is obvious that the cells are marked on the outer membrane with each MAB class. The calculated number of antigens per cell surface is about 4000 for class 1 MABs, 800 for class 2 and 3 MABs, and 100 for class 4 MABs. The numbers of antigen per cell surface were determined by calculating the average number of gold particles per ultrathin sectioned bacteria and the approximation that



FIG. 2. Biochemical characterization of the antigenic epitopes for the *Azospirillum brasilense* Sp245-specific MABs. (a) 1D-PAGE of a lipopolysaccharide preparation of *Azospirillum brasilense* Sp245. The gel was silver-stained. (b)-(e) Western blot of a 1D-PAGE lipopolysaccharide preparation of *Azospirillum brasilense* Sp245 (a) and immunoanalysis with MABs of classes 1-4 (Table 3).

TABLE 3: DIVISION OF THE MABs MIPE 3-1.1 - MIPE 1-106 INTO 4 CLASSES ACCORDING TO THE WESTERNBLOTTING RESULTS (for details see text)

	Mipe 3-1.1	Mipe 3-1.2	Mipe 3-1.3	Mipe 3-1.4	Mipe 3-1.6	Mipe 3-1.7	Mipe 3-1.8	Mipe 3-1.10	Mipe 3-1.11	Mipe 3-1.12	Mipe 1-103	Mipe 3-104	Mipe 1-105	Mipe 1-106
Class 1	+	-	-	-	-	-	+	-	-	-	-	-	-	-
Class 2	-	-	+	+	+	-	-	-	-	-	-	+	-	+
Class 3	+	+	-	-	-	+	-	+	+	+	-	-	-	-
Class 4	-	-	-	-	-	-	-	+	+	+	+	+	+	+

the bacterial cell is a cylinder 1  $\mu\text{m}$  in length and 0.5  $\mu\text{m}$  in width.

#### Determination of high affinity MABs

To determine the MAB with the highest affinity in each class a binding experiment (displacement study) was used to compare the affinity of MABs that bind to the same antigen. The results are shown in Figure 4A-D. Both MABs of class 1 have the same affinity. After the addition of 50 ng of MAB Mipe 3-1.1 to 50 ng bound MAB Mipe 3-1.8, 50% of the bound antibody was displaced. As a control experiment MAB Mipe 3-1.1 was displaced by MAB Mipe 3-1.8 (data not shown). The results did not change significantly. The same type of experiment was performed with MABs of class 2; MAB Mipe 3-1.3 showed the highest affinity levels. The 20-fold concentration of MAB Mipe-104 compared to the MAB Mipe 3-1.3 could not displace MAB Mipe 3-1.3 for more than 50%. The MAB with the highest affinity of class 3 was MAB Mipe 3-1.7. All IgM MABs of class 4 (Mipe-103, Mipe-105, Mipe-106) were of remarkably low affinity.

#### Validation of the MABs for a quantitative immunoassay

The validation of the MABs with the highest affinity in each class for a quantitative immunoassay is shown in Figure 5. Using an overnight culture of *Azospirillum brasilense* Sp245 (with *Azospirillum brasilense* Sp7 as control), a peroxidase-coupled secondary antibody, luminol as substrate, and a luminometric detection system, a quantification is possible with at least  $5 \times 10^2$  bacteria/ml (MAB Mipe 1.8). The detection limit for class 2 (MAB Mipe 3-1.3) and class 3 (MAB Mipe 3-1.7) was about  $10^4$  bacteria/ml. For class 4 (MAB 3.1.12) the detection limit was more than  $10^5$  bacteria/ml.

## DISCUSSION

With the use of ELISA techniques, western blotting of outer membrane components, and immunogold studies, we could identify mostly strain-specific MABs for *Azospirillum brasilense* Sp245. Some MABs were found to cross-react with

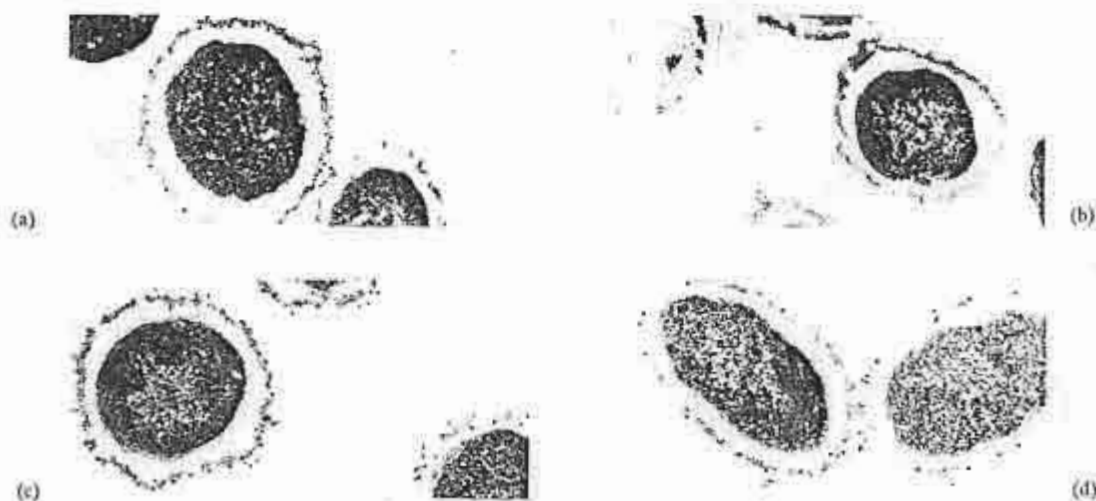


FIG. 3. Localization and quantification of the MAB epitopes by immunogold labeling of *Azospirillum brasilense* Sp245 cells and TEM (bar 0.5  $\mu\text{m}$ ). (a) *Azospirillum brasilense* Sp245 cells and immunogold labeling with class 1 MABs (Table 3). (b) *Azospirillum brasilense* Sp245 cells and immunogold labeling with class 2 MABs (Table 3). (c) *Azospirillum brasilense* Sp245 cells and immunogold labeling with class 3 MABs (Table 3). (d) *Azospirillum brasilense* Sp245 cells and immunogold labeling with class 4 MABs (Table 3).

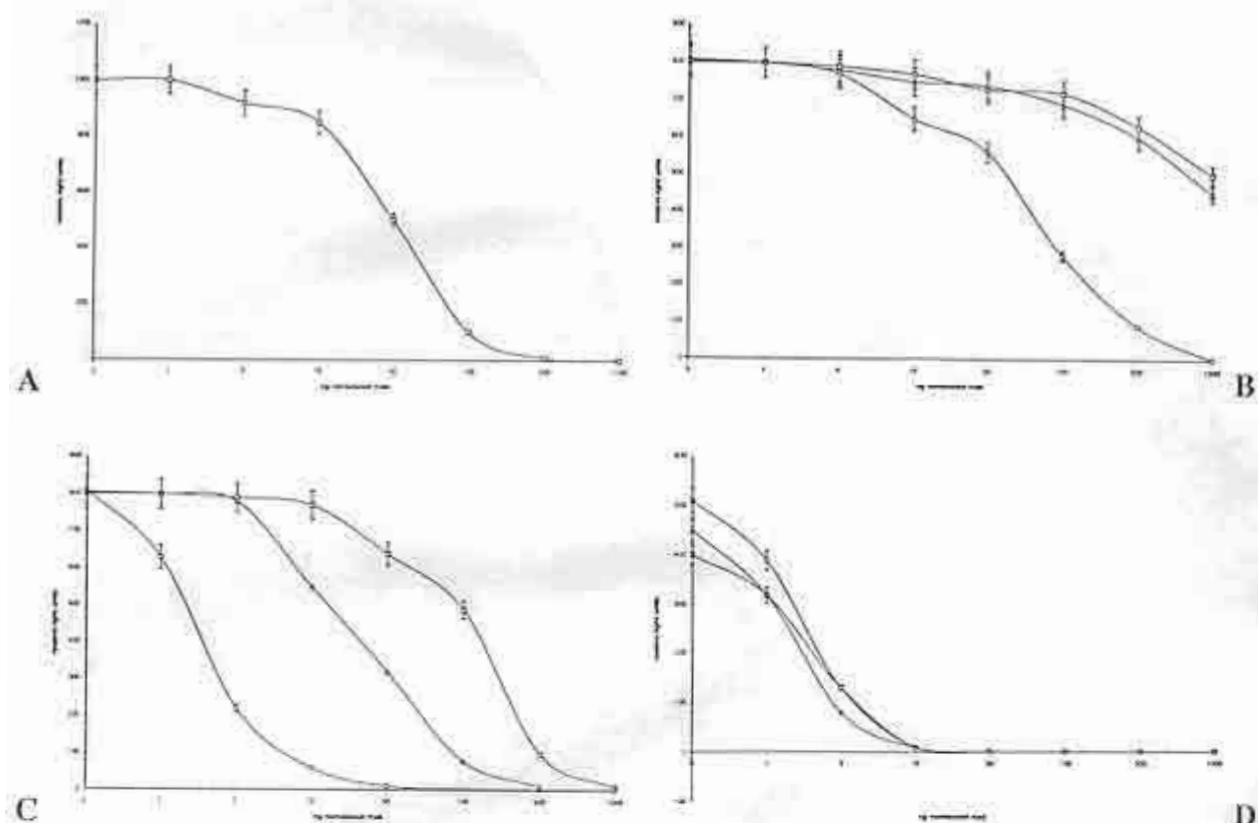


FIG. 4. Determination of the MAb affinity by displacement studies. A nonlabeled MAb competes with a peroxidase (POD)-labeled MAb for the antigen-binding site in ELISA. The antibody concentration of the labeled antibody was 50 ng/well. All experiments were done in 7 parallels/experiment. (A) Displacement of MAB 8—POD ( $\square$ ) by MAB 1 (class 1). (B) Displacement of MAB 3—POD ( $\square$ ), MAB 4—POD ( $\circ$ ), and MAB 6—POD ( $\Delta$ ) by MAB 104 (class 2). (C) Displacement of MAB 7—POD ( $\square$ ), MAB 10—POD ( $\circ$ ), and MAB 11—POD ( $\Delta$ ) by MAB 12 (class 3). (D) Displacement of MAB 103—POD ( $\square$ ), MAB 105—POD ( $\circ$ ), and MAB 106—POD ( $\Delta$ ) by MAB 12 (class 4).

*Azospirillum brasilense* Sp246, which is not surprising as both strains are closely related<sup>(22)</sup> and were isolated from the same wheat cultivar.<sup>(16)</sup> No other cross-reactions were determined. Bacteria of the species *Azospirillum brasilense* are very diverse in their outer membrane composition compared to other species. Using 2D electrophoresis of outer membrane proteins Schlöter *et al.*<sup>(23)</sup> showed, that most strains of *Azospirillum brasilense* have no outer membrane protein in common. Therefore, it is not surprising that most of the MAbs obtained were strain specific. In a previous publication it was shown that MAbs binding to outer membrane components of another *Azospirillum brasilense* strain (*Azospirillum brasilense* Sp7) were also strain specific, except for a cross-reaction with a very closely related strain (*Azospirillum brasilense* SpCd).<sup>(23)</sup> Although polyclonal antisera are a mixture of different antibodies, most of the sera for *Azospirillum brasilense*<sup>(26)</sup> were also strain specific. It is still an open question whether the high variability of cell surface components of *Azospirillum brasilense* is an indication of their ecological importance in adaptation to different soil and rhizosphere environments.

It was demonstrated that three different outer membrane proteins carry the antigenic determinants for class 2, class 3, and class 4 MAbs. However, no information about the function of

the proteins is available, because we still have no mutants that fail to bind to the different MAbs. The antigenic determinant for class 1 MAbs was LPS, which is common to *Azospirillum brasilense* Sp245 and *Azospirillum brasilense* Sp246. The surface localization of all MAbs was corroborated by immunogold labeling. The immunogold results also gave information about the number of antigenic determinants per cell. The calculated numbers correlate with the detection sensitivity of the MAbs. The MAbs with the highest number of antigenic determinants per cell (class 1) were most sensitive ( $5 \times 10^2$  bacteria/ml). The MAbs with the lowest number of antigenic determinants per cell (class 4) were least sensitive ( $1 \times 10^5$  bacteria/ml). The detection limits of mainly class 1 MAbs are much higher compared to polyclonal antisera, which are about  $10^5$  bacteria/ml.<sup>(26)</sup>

In every class high affinity MAbs were detected, which are most suitable for a sensitive detection of bacteria in complex ecosystems.

To use immunological methods for the localization and quantification of bacteria in complex habitats like the rhizosphere the antibodies must comply with four quality criteria: (1) localization of the antigenic determinant on the cell surface, (2) no cross-reaction, (3) stability of the antigenic determinant *in situ*, and (4) high affinity to the antigen (for a review see

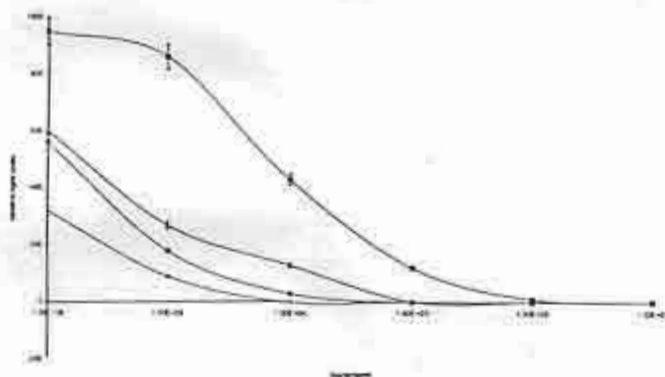


FIG. 5. Validation of MAb Mipe 1.8 (class 1) (■), MAb Mipe 3-1.3 (class 2) (●), MAb Mipe 3-1.7 (class 3) (▲), and MAb Mipe 3.1.12 (class 4) (—). Dilutions of *Azospirillum brasilense* Sp245 were subjected to quantitative chemoluminescence immunoassay (7 parallels/dilution); the light counts were measured in a microtiter plate luminometer.

Schlöter *et al.*<sup>(27)</sup>. In this work MABs that fulfill most of the quality criteria were obtained. Preliminary experiments on the stability of the different antigenic determinants under different ecological conditions were made. It seems that mainly the antigenic determinants of class 2 and 3 MABs are quantitative stable under rhizosphere conditions. The number of antigens for class 1 MABs seems to vary between rich medium and rhizosphere conditions. Further experiments on the stability are in progress and will be published elsewhere.

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