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Characterization of Monoclonal Antibodies Against Cell Surface Structures of *Azospirillum brasilense Sp7* Using ELISA Techniques

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Abstract

Hybridoma cell lines were generated using whole cells of *Azospirillum brasilense* Sp7. ELISA techniques were used to identify the specificity of the monoclonal antibodies (mabs). We obtained strain specific mabs directed against *Azospirillum brasilense* Sp7, but also mabs which cross-reacted with other bacteria. The binding sites of the mabs were characterized by competition chemoluminescence ELISA and cell surface mutants of *Azospirillum brasilense* Sp7. One class of mabs specifically bound to the polar flagellum of *Azospirillum brasilense* Sp7 and the closely related strain Cd.

Keywords: strain specific monoclonal antibodies, *Azospirillum brasilense*, outer membrane proteins, outer membrane polysaccharides, ELISA

1. Introduction

Cell surfaces play an important role in cellular adhesion, recognition and the perception and transduction of extracellular signals (Franzier and Glaser, 1979). In the case of the plant growth promoting *Azospirillum* spp., cell surface structures mediate the attachment to root cells (Michiels et al., 1989). In *Azospirillum brasilense* Sp7 a plasmid coded function was characterized which affects adsorption to wheat roots (Croes et al., 1991). Deletion mutants in

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this function apparently lack functional polar flagella. Michiels et al. (1991) demonstrated that a first rapid and weak adsorption process of *Azospirillum brasilense* Sp7 to wheat roots is abolished in this type of mutant. A second firm anchoring step of adsorbed bacteria is mediated by surface exopolysaccharides (EPS). Mutants deficient or altered in surface polysaccharide structure which bound less calcofluor white are lacking or reduced in this anchoring process (Michiels et al., 1991). *Azospirillum brasilense* Sp7 also produces an EPS which is secreted from the cells. It affects flocculation of the cells and lectin binding (Del Gallo et al., 1989). Some EPS mutants of *Azospirillum brasilense* complement *Rhizobium meliloti* *exoB* and *exoC* mutants (Michiels et al., 1988). Therefore, tools for a specific characterization of cell surface structures of *Azospirillum* are necessary for studies of the interaction of bacterial and root surfaces.

Since the outer membrane and cell surface of Gram-negative bacteria is a complex lipid bilayer containing glycoproteins, lipopolysaccharides (LPS), proteins and EPS, we decided to use the hybridoma technology to obtain monoclonal antibodies (mab) to specific bacterial cell surface epitopes. This approach has been used previously to examine, for example, the cell surface antigens of a number of bacterial phytopathogens (De Boer and Wieczorek, 1984; Alvarez et al., 1985). In immunological studies of *Azospirillum* until now only polyclonal antisera with different degrees of specifity have been used. De-Polli et al. (1980) could differentiate *Azospirillum* spp. serologically into distinct groups. Using live cells to raise antibodies in rabbits, Levanony et al. (1987) obtained a polyclonal serum, which showed high specificity towards *Azospirillum brasilense* Cd after purification of the gamma-globulin fraction. They demonstrated strain-specific antigens in its exopolysaccharide surface layer with immuno-gold staining. However, they did not identify the antigenic determinants (Levanony and Bashan, 1989).

The enzyme-linked immunosorbent assay (ELISA) is the most commonly used technique to characterize antibodies for a specific identification of bacteria (Clark, 1981). Cross-reaction experiments or the use of two different mabs in competition experiments provide a classification of the mabs in separate groups with different specificities and antigenic epitopes. Using specific cell surface mutants and flagellum preparations, some of the epitopes could be identified.

2. Materials and Methods

Bacterial strains

The bacterial strains were obtained from the German Collection of Microorganisms, Braunschweig, FRG, except *Azospirillum brasilense* wa3, which was kindly provided by C. Christiansen-Wenirger, Wageningen, The Netherlands, and *Azospirillum brasilense* Sp245 and Sp246, which were kindly provided by J. Döbereiner, Rio de Janeiro, Brazil.

The exopolysaccharide mutant *Azospirillum brasilense exoB217:Tn5*, the calcofluor binding mutant *Azospirillum brasilense 7030 Tn5-21* and the mutant *Azospirillum brasilense* p90Δ084, lacking polar flagella, were kindly provided by K. Michiels, Leuven, Belgium.

Mab production

The antibodies were obtained by immunization of 6–8 week old female mice (BALB/c) with living bacteria (Galfre and Milstein, 1981). *Azospirillum brasilense* Sp7 was grown in Luria broth medium at 33°C until the late exponential growth phase. A 0.5 ml aliquot was centrifuged and resuspended in phosphate buffered saline (PBS) to an absorbance (436 nm) of 1.0. The injections were performed intraperitoneally at three-week intervals. The spleen of one mouse was taken 4 days after immunization, homogenized and fused with the myeloma cell line x63-Ag8.653 in the presence of polyethylenglycol (PEG; Boehringer, FRG). The growing hybridoma cells were cultivated in 96-well microtiter plates (Nunc, FRG) in a medium containing RPMI 1640 (Biochrom, FRG) and 10% fetal calf serum (FCS, Biochrom FRG). After 14 days, growing clones were tested for antibody production using whole cells of *Azospirillum brasilense* Sp7 by indirect ELISA. Positive hybridomas were recloned and tested several times for antibody production. Productive cell lines were cultivated in 50 ml cell culture bottles to obtain antibodies on a larger scale. The obtained mabs were purified by hydroxylapatite column chromatography (Stanker et al., 1983).

Enzyme linked immunosorbent assay (ELISA) and competition chemoluminescence-ELISA

All immunoassays (IA) were performed with 96-well PVC microtiter plates (Flow, FRG) according to Harlow and Lane (1988), Niederwöhrmeier and Böhm (1990) and Schloter et al. (1992). To minimize crosslight effects, the bottom of the plates for the chemoluminescence-ELISA was painted with white colour. An overnight culture of the bacteria was centrifuged and resuspended

in carbonate buffer pH 9.6 to an absorbance at 436 nm of 1.0 (ELISA) or 0.1 (chemoluminescence-ELISA). Each well of the microtiter plates was filled with 50 μl of the bacterial suspension. All plates were incubated overnight at 4°C. Each well was washed with 150 μl of a solution containing 50% phosphate buffered saline, 0.5% FCS and 0.001% bovine serum albumin (Serva, FRG). To prevent nonspecific binding of antibodies to the PVC, the IA-plates were incubated with blocking solution (200 μl per well) containing 3% bovine serum albumin for half an hour at 37°C. After washing the plates, the mab (ELISA) or mixture of mabs (competition chemoluminescence-ELISA) was added. After incubation for 30 min at 37°C, the wells were washed three times. In the following step, 50 μl per well of goat antimouse IgG conjugated with horseradish peroxidase (Amersham, FRG) was added (diluted 1:400 in washing solution). After an incubation time of 45 min at 37°C, the plates were washed five times. The colorimetric test (ELISA) was started by adding 100 μl per well azino-di-[3-ethylbenzthiazoline] sulfonic acid (ABTS) as substrate in ABTS buffer (Boehringer, FRG). The absorbance at 405 nm was measured in an ELISA-plate photometer (Dynatech, FRG). The chemoluminescence-ELISA was started by adding 100 μl luminol (Amersham, FRG). The chemoluminescence was quantified in a microtiter plate luminometer (Dynatech, FRG).

Polar flagellum preparation

The flagella were prepared from 250 ml overnight culture of *Azospirillum brasilense* Sp7. The bacteria were centrifuged and resuspended in 15 ml 0.1 M Tris-HCl, pH 8. The flagella were separated from the bacteria by shredding in a waring blender at 19,500 rev/min for 45 sec. The cells were spun down by centrifugation for 10 min at 12000×g. The flagella were obtained from the supernatant by centrifugation at 55000×g for 30 min. The pellet was resuspended in water and subjected to a centrifugation in sucrose at 30000×g for 24 hr according to De Pamphilis and Adler (1971). Under these conditions, polar and lateral flagella could be separated.

3. Results

Initially, the mice were immunized with live cells of *Azospirillum brasilense* Sp7. Secreting hybridoma cell lines were screened for the production of antibodies directed against *Azospirillum brasilense* Sp7 using the indirect ELISA technique. Twenty-seven different mab-producing cell lines were identified in this way. The mabs were further characterized for their

properties to bind to different *Azospirillum* strains and other bacteria using the ELISA technique. Table 1 shows that three different classes of antibodies were revealed from these experiments. Antibodies in class 1 reacted with all species of *Azospirillum* and all other tested bacteria. Antibodies of class 2 bound to *Azospirillum brasilense* Sp7 and the very closely related strain *Azospirillum brasilense* Cd. Antibodies of class 3 were strain specific for *Azospirillum brasilense* Sp7, which was used as immunogen. According to this classification we obtained 9 mabs in class one, 4 mabs in class 2 and 14 mabs in class 3.

The chemoluminescence-ELISA was used for competition experiments in which two different mabs were added simultaneously at a high concentration to a small number of *Azospirillum brasilense* Sp7. If two mabs recognize different antigenic structures, the resulting chemoluminescence value is higher as compared to a single mab addition. According to this type of experiments, all three classes of mabs recognized different epitopes on the surface of the *Azospirillum brasilense* Sp7 cell. Mabs of class 1 recognized the same epitope. Mabs of class 2 reacted the same way. The mabs of class 3, however, appeared to have at least two different binding sites on the cell surface. Table 2 shows the results of the competition chemoluminescence-ELISA for class 3 antibodies, which clearly suggested to divide class 3 antibodies in two subclasses (3.1 and 3.2). We obtained 9 mabs of class 3.1 and 5 mabs of class 3.2.

Table 1. Comparison of the binding activity of different classes of mabs to different *Azospirillum brasilense* strains, *Azospirillum* species and other bacteria using the ELISA technique

Azospirillum strains	mab-classes		
	class 1	class 2	class 3
A. brasilense Sp7	+	+	+
A. brasilense Cd	+	+	–
A. brasilense Sp245	+	–	–
A. brasilense Sp246	+	–	–
A. brasilense wa3	+	–	–
A. lipoferum SpRG20a	+	–	–
A. amazonense Y1	+	–	–
A. halopreferens Au4	+	–	–
Pseudomonas fluorescence DSM 50090	+	–	–
Agrobacterium rubi DSM 30149	+	–	–
Bacillus subtilis DSM 10	+	–	–

"+" indicates binding of the mab
"–" indicates no binding of the mab

Table 2. Differentiation of class 3 mabs by competition-chemoluminescence-ELISA

	4	5	8	9	10	12	13	14	17	18	21	25	26	27
4	0	+	+	+	0	+	+	0	+	0	0	+	+	+
5	+	0	0	0	+	0	0	+	0	+	+	0	0	0
8	+	0	0	0	+	0	0	+	0	+	+	0	0	0
9	+	0	0	0	+	0	0	+	0	+	+	0	0	0
10	0	+	+	+	0	+	+	0	+	0	0	+	+	+
12	+	0	0	0	+	0	0	+	0	+	+	0	0	0
13	+	0	0	0	+	0	0	+	0	+	+	0	0	0
14	0	+	+	+	0	+	+	0	+	0	0	+	+	+
17	+	0	0	0	+	0	0	+	0	+	+	0	0	0
18	0	+	+	+	0	+	+	0	+	0	0	+	+	+
21	0	+	+	+	0	+	+	0	+	0	0	+	+	+
25	+	0	0	0	+	0	0	+	0	+	+	0	0	0
26	+	0	0	0	+	0	0	+	0	+	+	0	0	0
27	+	0	0	0	+	0	0	+	0	+	+	0	0	0

"+" indicates double signal strength in the competition chemoluminescence-ELISA
"0" indicates single signal strength in the competition chemoluminescence-ELISA

Using *Azospirillum brasilense* mutants altered in surface components, a first characterization of the antigenic structures was possible (Table 3). The *exoB*-mutation did not affect the binding of the mabs. The mabs of class 2 did not bind to the *Azospirillum brasilense* mutant p90Δ084, which has no polar flagella. The calcofluor binding mutant *Azospirillum brasilense 7030 Tn5-21*, which is altered in surface polysaccharide structure, did not bind the mabs of class 2 and 3.1.

Using a preparation of polar flagella of *Azospirillum brasilense* Sp7 as antigen

Table 3. Comparison of the binding activity of the different classes of mabs to different *Azospirillum brasilense* mutants using the ELISA technique

Azospirillum strains	mab classes			
	class 1	class 2	class 3.1	class 3.2
A. brasilense Sp7	+	+	+	+
A. bra Sp7 *exoB217:Tn5*	+	+	+	+
A. bra p90Δ084	+	–	+	+
A. bra 7030 Tn5-21	+	–	–	+

"+" indicates binding of the mab
"–" indicates no binding of the mab

in the ELISA test, a strong signal was obtained with mabs of class 2 only (data not shown). Lateral flagella gave no response. This result corroborates that mabs of class 2 specifically bind to the polar flagellum.

4. Discussion

Using live *Azospirillum brasilense* Sp7 cells as immunogen without further treatment, non-specific as well as specific monoclonal antibodies were obtained. Concerning the unspecific binding of class 1 mabs, we observed that the binding of these mabs to bacterial cells in ELISA experiments was less strong as compared to the binding of class 2 and 3 mabs, which recognize highly specific antigenic determinants. This could be taken as a hint to understanding the low selectivity of binding of class 1 mabs to unspecific sites.

The lack of binding of class 2 mabs to the *Azospirillum brasilense* motility mutant p90Δ084 as well as the binding of class 2 mabs to purified polar flagellin of *Azospirillum brasilense* Sp7 strongly suggest, that a component of the polar flagellum is the antigenic determinant for mabs of class 2. It is very interesting that antibodies directed against the polar flagellum only cross-react with the very closely related *Azospirillum brasilense* Cd. Apparently, all other *Azospirillum brasilense* strains have different antigenic determinants on their protein subunits of the polar flagellum. It is an open question, whether this finding has also ecological relevance for the interaction with plant surfaces.

Since calcofluor binds to β-1,2-glucans, the lack of binding of class 3.1 mabs to the calcofluor binding deficient mutant suggests exopolysaccharides as the antigenic determinant for this class of mabs. It is well known, that EPS components are highly immunogenic structures on the cell surface of Gram-negative bacteria (Brewin et al., 1986; Wingate et al., 1990). The immuno-gold labeling experiments of Levanony and Bashan (1989), who used a strain specific polyclonal IgG preparation, also indicate EPS as a possible antigen. However, since calcofluor-binding mutants are highly pleiotrophic (K. Michiels, pers. commun.), these mutants could also be affected in other surface properties. This may explain, why class 2 mabs also fail to bind to the calcofluor binding mutant (Table 3).

Until now, no mutants were found which were affected in binding of the class 3.2 mabs. This component may be of vital importance for *Azospirillum*. Electrophoresis and western blot experiments are in progress to further characterize the antigenic determinants.

Some of our mabs are directed towards cell surface structures, which are potential candidates for bacterial self-aggregation and bacteria root interaction.

Class 2 mabs, which specifically recognize the polar flagellum, are proper tools to further investigate the adsorption of *Azospirillum brasilense* Sp7 to wheat roots, because mutants lacking the polar flagellum fail to adsorb rapidly to roots (Michiels et al., 1991). Class 3.1 mabs, which are directed against an exopolysaccharide, can be used to proof their role in the anchoring of *Azospirillum brasilense* Sp7 to the root surface by antibody blocking experiments.

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