

Characterization of cell surface components of *Azospirillum brasilense* Sp7 as antigenic determinants for strain-specific monoclonal antibodies

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Monoclonal antibodies (mAbs) with high specificity for *Azospirillum brasilense* Sp7, and which bind to different antigenic determinants, were characterized using Western blot techniques applied to one- and two-dimensional fingerprints of the outer-membrane components, and by immunogold labelling combined with transmission electron microscopy. One class of mAbs, which bound to *A. brasilense* Sp7 and the closely related strain Cd, recognized a 100 kDa protein subunit of the polar flagellum. Two classes of strain-specific mAbs for *A. brasilense* Sp7 bound, respectively, to a 85 kDa outer-membrane protein and to polysaccharide.

Keywords: *Azospirillum brasilense*, cell surface, monoclonal antibodies, outer-membrane proteins, plant–microbe interactions

INTRODUCTION

Some bacteria of the genus *Azospirillum* have plant-growth-promoting potential (Döbereiner & Pedrosa, 1987; Okon, 1985). Important steps to establish this rhizocoenosis are the adsorption of bacteria to the root surface and the colonization of the root. It is known that different protein or carbohydrate components of the bacterial cell surface are involved in attachment to plant surfaces (Diaz *et al.*, 1989; Vesper & Bauer, 1986; Whatley *et al.*, 1976). Lectin- and calcofluor-binding exocellular polysaccharides as well as surface proteins may be involved in the *Azospirillum* – root interaction (Del Gallo *et al.*, 1989; Michiels *et al.*, 1989). Some of the bacterial components involved in the interaction with root surfaces also play a role in self-aggregation and flocculation (Madi & Henis, 1989; Michiels *et al.*, 1991). Michiels *et al.* (1991) demonstrated that attachment of *A. brasilense* Sp7 to wheat roots occurs in two steps. The first step, a rapid and weak adsorption process, was abolished in a non-flagellated mutant, which was isolated by deletion mutagenesis of the 90 MDa plasmid of *A. brasilense* Sp7. This plasmid was proposed as a rhizocoenotic plasmid (Croes *et al.*, 1991). The second step, a firm anchoring of adsorbed bacteria, is probably mediated by surface polysaccharides

(exopolysaccharides or lipopolysaccharides). The involvement of the polar flagellum in the *Azospirillum*–root adsorption was recently substantiated by Croes *et al.* (1993). Mutants with a deficient or altered surface polysaccharide structure, as shown by altered binding of calcofluor white to the mutant bacteria, were impaired in the anchoring process (Michiels *et al.*, 1990).

To develop specific tools to study the molecular interaction of *A. brasilense* with roots, strain-specific monoclonal antibodies (mAbs) were isolated which bind to cell surface structures. Using the competition ELISA technique with mutants impaired in cell surface structures, 27 mAbs were divided into three classes (Schloter *et al.*, 1992). Class 1 mAbs cross-reacted with many bacteria. The class 2 mAbs reacted only with *A. brasilense* Sp7 and the closely related strain Cd. The third class of mAbs bound specifically to *A. brasilense* Sp7. Using a competition ELISA, class 3 mAbs were divided into the subclasses 3.1 and 3.2. (Schloter *et al.*, 1992).

In this study, we used one-(1-D) and two-dimensional (2-D) PAGE to separate outer-membrane and cell surface components of *A. brasilense*. The 2-D fingerprint was recently described by De Mot & Vanderleyden (1989) as a strain-specific method to identify *Azospirillum* strains. The cell surface components to which the different types of mAbs bind were subsequently identified by Western

Abbreviations: mAb, monoclonal antibody; 1-D, 2-D, one-, two-dimensional.

blot analysis. The cellular localization of the antigenic determinants was further investigated by transmission electron microscopy of immunogold-labelled *A. brasilense* Sp7 cells.

METHODS

Bacterial strains and growth conditions. *A. brasilense* Sp7 was obtained from the German Collection of Microorganisms (DSM), Braunschweig, Germany. The mutant *A. brasilense* Sp7 p90D084, which is non-motile and lacks the polar flagellum, has been described recently (Croes *et al.*, 1991, 1993). The bacteria were grown in Luria broth (LB) medium or 3% trypticase soy broth at 33 °C until early stationary phase.

Monoclonal antibodies. mAb-producing hybridoma cell lines were obtained by fusion of the myeloma cell line X63-Ag8.653 with B-lymphocytes of 1–4-month-old mice, which had been stimulated 1–5 times by immunization with live cells of *A. brasilense* Sp7 (Galfre & Milstein, 1981). mAbs with high specificity for *A. brasilense* Sp7 were purified by hydroxylapatite column chromatography (Stanker *et al.*, 1983). The present experiments are carried out with one mAb from each class: Mic 2-4.2 (class 2), Mic 4-1.2 (class 3.1) and Mic 3-8.1 (class 3.2).

Isolation of *A. brasilense* polar and lateral flagella. We adopted the procedure described by De Pamphilis & Adler (1971) with some modifications. Portions (1 ml) of a fresh *A. brasilense* culture were spread on fifty 10 cm × 10 cm square Petri dishes containing LB solidified with 1.5% (w/v) agar. After 48 h incubation at 30 °C in a humid atmosphere, cells were collected in 5 ml 10 mM Tris/HCl (pH 8.0) per plate. The bacteria were pelleted by centrifugation (4×10^3 g, 15 min) and resuspended in 100 ml of the same buffer. This suspension was mixed for 40 s at maximum speed in a Waring Blender mixer to break the flagella. The bacteria and cell debris were spun down from the mixed cell suspension for 15 min at 10^4 g. From the supernatant liquid, flagella were pelleted by ultracentrifugation for 90 min at 2×10^4 g and resuspended in 1 ml of sterile water to obtain the crude flagellar suspension. For further purification and separation of polar and lateral flagella, 13.4 g dried CsCl was added and the volume of the suspension was adjusted to 30 ml with 10 mM Tris/HCl (pH 8.0). A density gradient was then established by spinning the samples for 60 h at 2×10^4 g in a Beckman SW28 rotor. Opaescent bands were carefully removed from the gradient with a Pasteur pipette, dialysed against water, lyophilized, and resuspended in 40 µl water.

Isolation of outer-membrane proteins. Outer-membrane components were isolated by the method described by Bachhawat & Gosh (1987) with some modifications. About 2×10^9 – 4×10^9 stationary-phase cells grown in 3% trypticase soy broth were washed twice with PBS (gl⁻¹: NaCl, 8; KCl, 0.2; Na₂HPO₄, 1.2; KH₂PO₄, 0.2; pH 7.2). The washed cells were sonicated for 6 min at 80 W. The unbroken cells were removed by centrifugation for 10 min at 10^3 g. The supernatant was centrifuged at 6×10^4 g for 30 min at 5 °C. The pellet, constituting the crude envelope fraction, was resuspended in 8 ml of a 10 mM Tris/HCl buffer (pH 8.0) containing 0.5% N-laurylsarcosine. After incubation for 30 min at 28 °C, the suspension was centrifuged at 5×10^4 g for 30 min at 5 °C. The pellet containing the outer-membrane components was resuspended in 100 µl 10 mM Tris/HCl buffer (pH 8.0). It was shown by Bachhawat & Gosh (1987) that this procedure yields an *A. brasilense* outer-membrane preparation which is not substantially contaminated with cytoplasmic or inner-membrane proteins (NADH oxidase and succinate dehydrogenase activity

was measured). Compared with the use of sucrose gradient centrifugation to isolate outer (and inner) membranes from the crude envelope fraction, this method resulted in identical patterns for *A. brasilense*, but had the advantage of being much simpler to perform. Subsequently, proteins were isolated from the outer membranes by phenol extraction (De Mot & Vanderleyden, 1989).

Lipopolysaccharide extraction. Outer membranes were prepared as described above using 1×10^8 stationary-phase cells (grown at 33 °C in LB), but the final phenol extraction was omitted. The lipopolysaccharide fraction was obtained by proteinase K treatment (25 mg ml⁻¹) at 37 °C overnight.

One- and two-dimensional gel electrophoresis. 1-D gels were performed as SDS-polyacrylamide-pore-gradient-gels (10–22%) with 4% stacking gels on a horizontal apparatus prepared according to Laemmli (1970) (1-D PAGE), using 0.375 M Tris/HCl (pH 8.8) as gel buffer. The dimensions of the gels were 30 × 20 × 0.02 cm. The gels were transferred for Western blotting or stained with Coomassie brilliant blue R250 followed by silver nitrate staining (Heukeshoven & Dernick, 1983).

2-D PAGE was carried out according to O'Farrell (1975) as described by De Mot & Vanderleyden (1989). The gels were transferred for Western blotting or stained with Coomassie brilliant blue R250.

Western blotting. 1-D or 2-D gels were electroblotted on Immobilon-P membranes (Millipore). Immunodetection was performed in combination with a horseradish peroxidase-conjugated goat-anti-mouse secondary antiserum and with 4-chloro-1-naphthol as a substrate to develop the blots (Harlow & Lane, 1988).

Immunogold labelling and transmission electron microscopy (TEM). Bacteria were collected in PBS from a fresh LB plate and diluted to a density of 10^8 cells ml⁻¹. The cells were washed twice with PBS containing 1% BSA, incubated for 1 h with 100 µl of the mAb, washed three times with PBS/0.02% BSA, incubated for 2 h with goat-anti-mouse gold conjugate (5 nm gold particles), and finally washed three times with PBS/0.02% BSA. Bacteria labelled with class 2 mAbs were stained for 1 min with 1% phosphotungstic acid (pH 7.0) on Formvar- and carbon-coated copper grids for TEM.

Bacteria labelled with class 3 mAbs were fixed for 10 min in 3% glutaraldehyde, washed once with PBS/0.02% BSA and embedded in LR white resin. Sections (70 nm) were cut with a microtome and were fixed on Formvar-coated copper grids for TEM.

RESULTS

mAbs of class 2 bind specifically to the *A. brasilense* Sp7 polar flagellum

A 2-D fingerprint of the cell surface proteins of *A. brasilense* Sp7 is shown in Fig. 1(a). Using a Western blot, the antigenic determinant of the class 2 mAbs, which cross-react with the closely related strains *A. brasilense* Sp7 and *A. brasilense* Cd, was identified as a 100 kDa protein with an isoelectric point of 3.5 (Fig. 1b). 2-D fingerprints of the cell surface proteins of the non-motile mutant *A. brasilense* Sp7 p90D084 revealed the absence in this mutant of the 100 kDa protein recognized by mAbs of class 2 (not shown).

Using CsCl gradient centrifugation, two bands of different

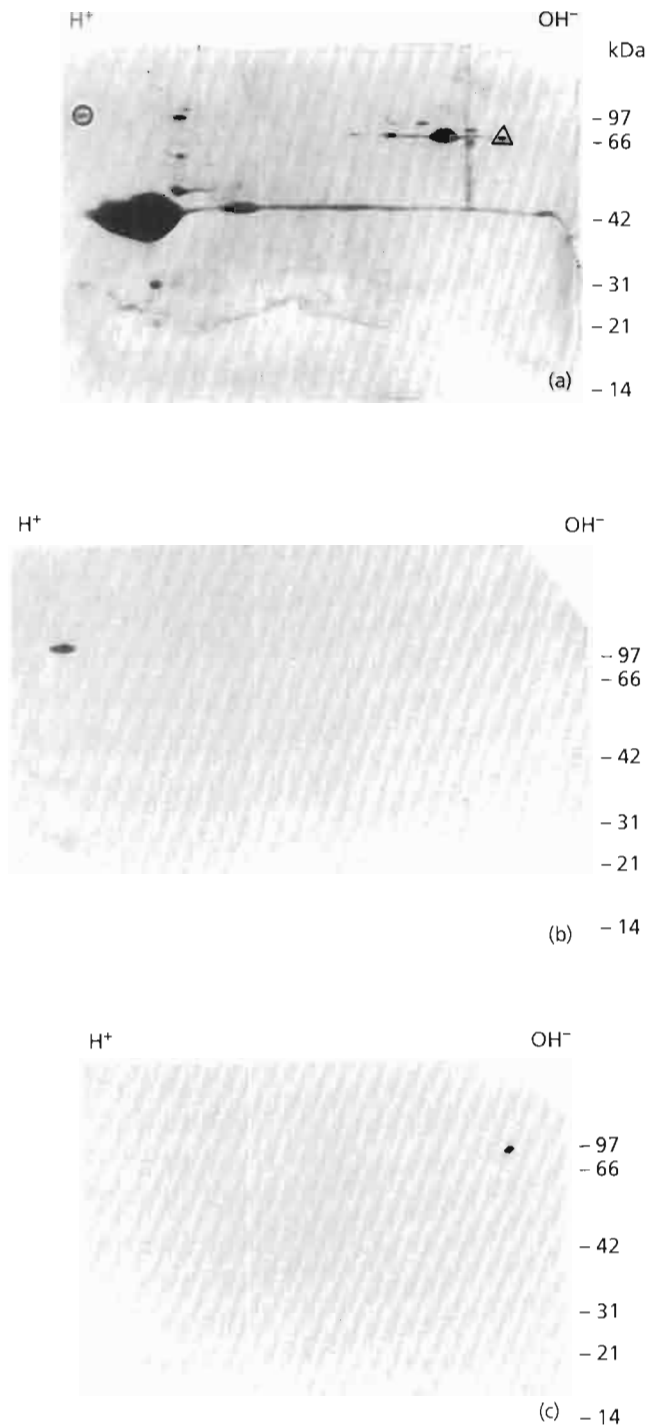


Fig. 1. Biochemical characterization of antigenic epitopes for the *A. brasilense* Sp7-specific mAbs. (a) 2-D fingerprint of the cell wall proteins of *A. brasilense* Sp7. The gel was stained with Coomassie Blue. (b) Western blot of a 2-D fingerprint of the cell wall proteins of *A. brasilense* Sp7 with class 2 antibodies. For detection, 4-chloro-1-naphthol was used. The corresponding protein in Fig. 1(a) is marked with a circle. (c) Western blot of a 2-D fingerprint of the cell wall proteins of *A. brasilense* Sp7 with class 3.2 antibodies. For detection, 4-chloro-1-naphthol was used. The corresponding protein in Fig. 1(a) is marked with a triangle. A pH range from 3.5 (H⁺) to 10 (OH⁻) was used for the isoelectric focusing. The estimated molecular masses of the standard proteins are shown.

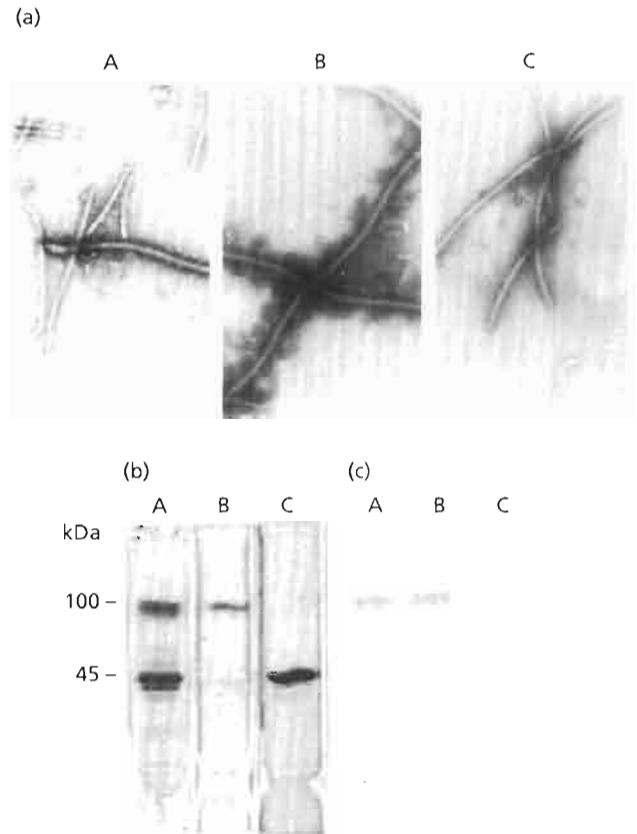


Fig. 2. Analysis of flagellar preparations by TEM (a), SDS-PAGE (b) and Western blotting with class 2 mAbs (c). A, Crude flagellar preparation before CsCl fractionation, containing both types of flagella; B, CsCl gradient lower band, containing the thick polar flagella; C, CsCl gradient upper band, containing the thin lateral flagella. Estimated molecular masses of the flagellins are given.

density were obtained from crude flagellar extracts. The crude extract and the two CsCl purified fractions were examined by TEM after negative staining, and by SDS-PAGE to identify the protein composition. TEM revealed fragments from two types of flagella differing in diameter. The high density CsCl fraction contained only the thick flagella, whereas the low density fraction contained the thin flagella (Fig. 2a). It is known that *A. brasilense* has a single polar flagellum, and produces multiple lateral flagella only in semi-solid or solid media. The latter are thinner and differ in antigenic properties from the polar flagellum (Hall & Krieg, 1983). Thus, by CsCl centrifugation, we obtained an efficient separation of both flagellum types. SDS-PAGE revealed that the polar flagella (i.e. the heavy CsCl fraction) consist mainly of a 100 kDa protein, whereas in the lateral flagella, a 45 kDa protein is predominant. These major proteins likely represent the flagellins of the respective flagellum types. Minor protein bands, like the 40 kDa band in both preparations, may represent other components of the flagellar structure or may be derived from the cell surface (Fig. 2b). A Western blot was prepared from a flagellar preparation



Fig. 3. Localization of the mAb epitopes by immunogold labelling of *A. brasilense* Sp7 cells and TEM. (a) A polar flagellum of *A. brasilense* Sp7 and immunogold labelling with class 2 mAbs. Bar, 0.5 µm. (b) *A. brasilense* Sp7 cells and immunogold labelling with class 3.1 mAbs. Bar, 5 µm. (c) *A. brasilense* Sp7 cells and immunogold labelling with class 3.2 mAbs. Bar, 5 µm.

after SDS-PAGE and developed with class 2 mAbs, showing that these mAbs reacted with the 100 kDa flagellin from the polar flagellum (Fig. 2c). The crude flagellar extracts of the non-motile mutant *A. brasilense*

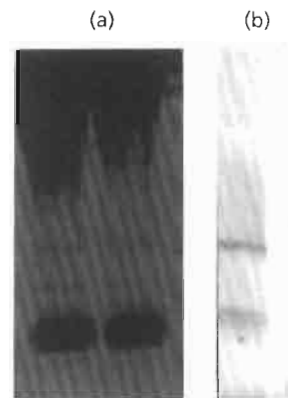


Fig. 4. Biochemical characterization of antigenic epitopes for the *A. brasilense* Sp7-specific class 3.1 mAbs. (a) 1-D SDS polyacrylamide gel of outer-membrane polysaccharides of *A. brasilense* Sp7. The gel was stained with silver. (b) Western blot of a 1-D SDS polyacrylamide gel of outer-membrane polysaccharides of *A. brasilense* Sp7 with class 3.1 mAbs. For detection, 4-chloro-1-naphthol was used.

Sp7 p90D084 did not yield any CsCl bands and did not contain the 45 kDa and 100 kDa flagellins (data not shown).

Binding of class 2 mAbs to the polar flagellum was confirmed by immunogold labelling and TEM studies (Fig. 3a). No binding of class 2 mAbs to the lateral flagella or the cell surface was observed. TEM studies of *A. brasilense* Sp7 p90D084 confirmed the results of lacking polar and lateral flagella (data not shown).

mAbs of class 3.1 bind specifically to lipopolysaccharides of *A. brasilense* Sp7

mAbs, which belong to the *A. brasilense* Sp7-specific class 3.1, gave no Western blot signal on a 2-D fingerprint of the outer-membrane proteins. *A. brasilense* mutants altered in calcofluor white binding did not react with mAbs of class 3.1 (Schlotter *et al.*, 1992), suggesting that these mAbs bind to a cell surface polysaccharide. A lipopolysaccharide extract of *A. brasilense* Sp7 was prepared and separated by SDS-PAGE, yielding a multiple band pattern upon silver staining (Fig. 4a). Since no bands were seen after the gel had been stained with Coomassie blue, all proteins had been destroyed by proteinase K treatment. A Western blot of this gel developed with mAbs of class 3.1 resulted in two bands (Fig. 4b).

The binding of class 3.1 mAbs to cell surface components was confirmed by immunogold labelling and TEM (Fig. 3b). As expected for a lipopolysaccharide-specific antibody, the gold label was distributed over the entire cell surface.

mAbs of class 3.2 bind specifically to a 85 kDa outer-membrane protein of *A. brasilense* Sp7

mAbs, which belong to the *A. brasilense* Sp7-specific class 3.2, gave a signal on a Western blot of a 2-D fingerprint

of the outer-membrane proteins of *A. brasilense* Sp7 with a basic 85 kDa protein (Fig. 1c).

Binding of class 3.2 mAbs to the cell surface was confirmed by immunogold labelling and TEM studies (Fig. 3c). The antigenic determinant is found at the cell surface.

DISCUSSION

By the use of Western blotting of 1-D or 2-D fingerprints of outer-membrane components and by immunogold studies, we have identified mostly strain-specific immunoreactive outer-membrane components of *A. brasilense* Sp7.

Three lines of evidence enabled the identification of the filament protein of the polar flagellum as the antigenic determinant for class 2 mAbs. Firstly, mAb binding to a 100 kDa protein on Western blots of a 2-D fingerprint of outer-membrane proteins. This protein was identified as the major constituent of a polar flagellum preparation by SDS-PAGE. Secondly, lack of mAb binding to the mutant *A. brasilense* Sp7 p90D084 in ELISA (Schloter *et al.*, 1992). This mutant is non-flagellate (Croes *et al.*, 1993) and lacks the 100 kDa protein. Thirdly, mAb binding to the polar flagellum was shown by immunogold labelling. Since the mutant *A. brasilense* Sp7 p90D084 which has a deletion in the rhizocoenotic 90 MDa plasmid, and other non-flagellate mutants, are unable to adsorb to the plant root surface (Michiels *et al.*, 1991; Croes *et al.*, 1993), the mAbs specific for the flagellum could be used in blocking experiments to independently demonstrate the involvement of flagella in the bacteria-root interaction. There is also evidence from a plant-growth-stimulating *Pseudomonas fluorescens* strain that flagella are required for colonization of potato roots (De Weger *et al.*, 1987).

While the mAbs directed against the flagella attached to the closely related strains Sp7 and Cd, the mAbs which bind to lipopolysaccharides recognize *A. brasilense* Sp7 specifically (Schloter *et al.*, 1992). Lipopolysaccharide binding was demonstrated by Western blotting of a lipopolysaccharide preparation and by immunogold studies. It is well known that different parts of the O-polysaccharides of the Gram-negative bacterial cell surface are very immunogenic (Lüderitz *et al.*, 1982). The variations in the outer core region and the O-polysaccharide antigen give rise to specific serotypes. Recently, mAbs against a relatively low molecular mass lipopolysaccharide of *Pseudomonas aeruginosa* were characterized (Yokota *et al.*, 1992). *Azospirillum* mutants altered in calcofluor white binding, self-aggregation and the anchoring process on wheat roots have been described (Michiels *et al.*, 1990, 1991). These mutants also failed to bind class 3.1 mAbs as demonstrated by ELISA techniques, suggesting that they have modified lipopolysaccharides (Schloter *et al.*, 1992). It would be very interesting to test the ability of *in vivo* inhibition of the anchoring process by these mAbs to assess the role of lipopolysaccharides in this process.

The 85 kDa outer-membrane protein was demonstrated

to carry the antigenic determinant for class 3.2 strain-specific mAbs and its surface location was corroborated by immunogold TEM investigations. However, no information about its function is available, because we do not have a mutant which fails to bind the mAbs.

Levanony & Bashan (1989) showed a surface localization of specific antigens both at the polar flagellum and the exopolysaccharide layer by immunogold-staining using a polyclonal antiserum. As a polyclonal serum is mixture of different antibodies, it can bind to different epitopes.

It is an open question whether the high variability of antigenic cell surface structures of *Azospirillum* is an indication of their ecological importance in adaptation to different soil and rhizosphere environments. It is most remarkable that *A. brasilense* strains Sp7 and Cd can be distinguished by mAbs against lipopolysaccharide and against the 85 kDa outer-membrane protein, since both strains are very closely related. Strain Cd was isolated from *Cynodon dactylon* roots in California after inoculation with strain Sp7 (Tarrand *et al.*, 1978). Strains Sp7 and Cd showed only very slight or no difference in restriction fragment length polymorphism, which usually allows a strain-specific identification (Fani *et al.*, 1991; Gündisch *et al.*, 1993). Moreover, protein fingerprinting by 2-D PAGE revealed only few differences in the total protein pattern of these strains (De Mot & Vanderleyden, 1989).

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