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EXPERIMENTAL ARTICLES

Analysis of DNA, Lipopolysaccharide Structure, and Some Cultural and Morphological Properties in Closely Related Strains of *Azospirillum brasilense*

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Abstract—We studied closely related *Azospirillum brasilense* strains Sp7 and Cd. For probing of their genomes, the fragments of 85 MDa (p85) and 120 MDa (p120) from *A. brasilense* Sp245 plasmids were hybridized with 115-MDa (p115) and 90-MDa (p90) plasmids of strain Sp7, respectively. Strain Cd was found to lose the 115-MDa plasmid and one of the two *Eco*RI restriction fragments of the total DNA (localized within p115 and the chromosome) that was homologous to an *Eco*RI-generated p85 fragment of 2.4 kb. On the contrary, in the total DNA of strain Sp7-S, in spite of the previously established disappearance of the 115-MDa replicon, two fragments homologous to p85 were revealed, as with strain Sp7. It is suggested that the Sp7-S genome contains the total p115 DNA or at least a certain part of it. Strains Sp7 and Cd were found to differ in size and morphology of colonies on solid and semisolid media, in the levels of resistance to the cation surfactant cetavlon, and in the antigen structure of lipopolysaccharides.

Key words: Azospirillum brasilense, plasmids, colony morphology, O-antigens.

Gram-negative alphaproteobacteria of the genus *Azospirillum* have been one of the primary objects in studies of mutually beneficial plant–microbial associations for the last 20 years. These bacteria have a large genome which includes chromosomal DNA and numerous large plasmids, which, presumably, play an important role in associative interactions [1].

The properties and functions of plasmid DNA have been studied most actively in two model strains of *Azospirillum brasilense*, Sp7 and Sp245 [1]. It was shown that a 120-MDa plasmid (p120) of strain Sp245 and a 90-MDa plasmid (p90, or pRhico) of strain Sp7 participate in regulatory mechanisms of the formation of exopolysaccharides (EPS), lipopolysaccharides (LPS, Lps), polar (Fla) and lateral (Laf) flagella, and swimming (Mot) and swarming (Swa) motility [1–3]. In a 85-MDa plasmid (p85) of strain Sp245, the *fla/laf/mot/swa* locus was identified. Mutations of this locus resulted in the loss of Fla and Laf (Fla^- Laf^- phenotype) or their paralysis (Mot^- Swa^- phenotype) [1]. In a 115-MDa plasmid (p115) of strain Sp7, a homologous locus was identified [4]. Also, regions homologous to the *fla/swa* locus and the *lps/cal* loci of p120 were found in pRhico. Inactivation of the *fla/swa* locus resulted in the Fla^- Swa^- phenotype, whereas mutagenesis of the *lps/cal* loci induced defects in the formation of LPS I or LPS II and polysaccharides stained with calcofluor (Cal^- phenotype) [1, 2, 4].

Functions of the 115-MDa plasmid (p115) of the type strain *A. brasilense* Sp7 are practically unknown. Matveev and coworkers [5] obtained indirect data on the possible role of p115 in the regulation of R–S dissociation in azospirilla. The loss of p115 by Sp7 cells correlated with persistence of the S phenotype, i.e., smooth colonies (a corresponding strain was named Sp7-S). DNA hybridization analysis was not used in [5]. It turned out that the difference between the R and S types in Sp7 results from changes in the contributions of two full-value LPSs to the architecture of the colony surface with culture ageing rather than from partial or complete loss of the O-specific polysaccharide (O-PS) by R-type cells [6]. After several years of storage at –70°C in glycerol-containing medium, strain Sp7-S was plated onto solid medium, and spontaneous S, RS, and R variants carrying a 82–131 MDa plasmid instead of p90 were selected ([7] and Petrova *et al.*, manuscript in preparation). Remarkably, a 131-MDa replicon of two R derivatives of strain Sp7-S had sites homologous to both p90 and p115 [7].

In contrast to *A. brasilense* Sp7 and Sp7-S and other azospirillum strains, strain Cd does not dissociate on minimal synthetic media and retains the S phenotype over a long period of time (see RESULTS AND DISCUSSION). However, strains Sp7 and Cd are closely related [8–13]. Strain Sp7 was isolated from the rhizosphere of *Digitaria decumbens*, whereas strain Cd was obtained from *Cynodon dactylon* roots after inoculation of the plant with Sp7 [8, 9]. These strains are

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Bacterial strains and plasmids used throughout this study

Strain or plasmid	Characteristics	Source
Strains:		
Azospirillum brasilense		
Sp7	Wild strain, isolated in Brazil from the rhizosphere of *Digitaria decumbens*	[8]
Cd	Wild strain, isolated in California from roots of *Cynodon dactylon* after inoculation with Sp7	[9]
Sp245	Wild strain, isolated in Brazil from wheat roots	[14]
Escherichia coli		
DH1	*supE44*, *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*, recipient of recombinant plasmids	[15]
Plasmids:		
pOmegon-Km-*lps*348X	*Xho*I-fragment of p120, carrying Omegon-Km (3.8 kb) and the *lps*/*cal* locus from *A. brasilense* KM348 (LpsI$^-$ mutant of Sp245), 18.8 kb, Km^R. A 15-kb *Bam*HI-fragment of this plasmid was used as a probe in reactions of DNA hybridization	[4]
pOmegon-Km-*fla*048X	*Xho*I-fragment of p120, carrying Omegon-Km and the *fla*/*swa* locus from *A. brasilense* SK048 (Fla$^-$ Swa$^-$ mutant of Sp245), 12.1 kb, Km^R. A 8.3-kb *Bam*HI-fragment of this plasmid was used as a probe in reactions of DNA hybridization	[4]
pEK051X	*Xho*I-fragment of the cointegrate p85::pJFF350 from *A. brasilense* SK051 (Fla$^-$ Laf$^-$ mutant of Sp245), 23 kb, Km^R. A 2.4-kb *Eco*RI-fragment of this cointegrate was used as a probe in reactions of DNA hybridization	[4]

characterized by practically identical two-dimensional protein maps [10] and length polymorphism of *Spe*I-fragments of DNA restriction [11], 100% homology of chromosomal DNA [8], and slight differences in length polymorphism (in the interval of 1.7–0.3 kb) of *Bgl*II-fragments of DNA restriction [12]. Monoclonal antibodies to polar flagella of strain Sp7 recognized flagella of strain Cd. However, monoclonal antibodies to the LPS and an 85-kDa protein of the outer membrane of Sp7 did not bind to Cd cells [13]. It is plausible to assume that differences in the cell surface antigens of strains Sp7 and Cd emerged in the process of adaptation to different environments [13]. We did not find more specific published data on the character of LPS changes in strain Cd. Information on genetic and immunochemical aspects of LPS modifications in azospirilla in the process of R–S dissociation is also very scarce.

This work was aimed at comparative analysis of DNA, some cultural and morphological properties, and LPS structure in closely related *A. brasilense* strains Sp7 and Cd, which differ in capability of R–S dissociation, as well as at estimating the possibility of differentiation between these strains with the use of molecular probes containing fragments of plasmids of strain Sp245.

MATERIALS AND METHODS

Plasmids, strains, and cultivation conditions. Bacterial strains and plasmids used throughout this study are described in the table. *Escherichia coli* was grown on an LB medium [15] at 37°C. *Azospirillum brasilense* was grown at 32°C on malate–salt medium [16] supplemented with 0.5 g/l NH_4Cl, on filtered potato broth (200 g of peeled and cut tubers per liter of water) (PM), and on medium MSM containing 2% glycerol instead of malate (GSM); pH of all media was adjusted to 6.8. When necessary, the media were supplemented with cationic surfactant cetyl trimethyl ammonium bromide (cetavlon, 0.01–0.1%), the anionic surfactant sodium dodecyl sulfate (SDS, 0.005–0.1%), and ampicillin (Ap, 50–1000 μg/ml) or kanamycin (Km, 50 μg/ml). The motility of azospirilla was judged from the diameter of rings formed after stab inoculation into MSM containing 0.3–0.4% Bacto agar.

Methods of LPS study. Polyclonal rabbit anti-LPS antibodies (Ab) were obtained as described earlier [17]. Double immunodiffusion, linear immunoelectrophoresis, LPS isolation, and LPS electrophoresis in polyacrylamide gel were carried out as described by Matora and coauthors [6, 17]. To obtain spectrophotometric characteristics of LPSs, we used a standard method based on the ability of carbohydrates to react with phenol with the formation of colored products after treatment with strong acid [18]. Absorption spectra of the colored products were recorded on a Specord M-40 spectrophotometer (Carl Zeiss, Germany).

Methods of DNA treatment. Plasmids were visualized by the Eckhardt method [19]. DNA isolation, purification, endonuclease restriction, and electroelution of DNA fragments from agarose gels were carried out by standard methods [15]. Labeling of DNA fragments with peroxidase and nonradioactive ECL-blotting-hybridization were conducted in accordance with recommendations of the manufacturer (Amersham Pharmacia Biosciences).

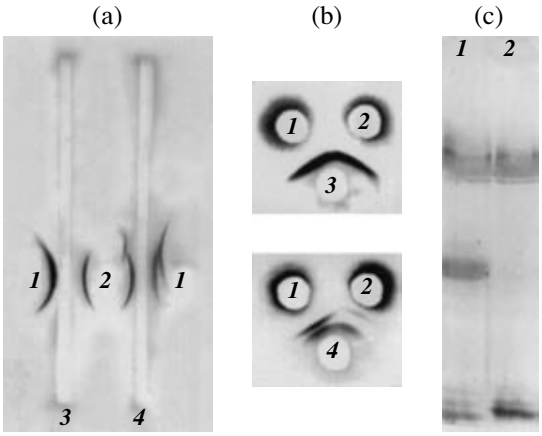

Fig. 1. (a) Linear immunoelectrophoresis, (b) double immunodiffusion, and (c) PAAG electrophoresis of the LPSs of *A. brasilense*: (*1*) strain Sp7, (*2*) strain Cd, (*3*) anti-Cd Ab, (*4*) anti-Sp7 Ab.

RESULTS AND DISCUSSION

Cultural and morphological characteristics of *A. brasilense* strains Sp7 and Cd. The cell surface is very dynamic and plays an important role in adaptation to different econiches, including plant–microbial associations. Modifications of the cell surface structures often result in changes of colony morphology on solid and semisolid media. The colony macrostructure of strains Sp7 and the derivative strain Cd was distinctively different on synthetic mineral medium. On MSM agar, *A. brasilense* Sp7 formed smooth and glossy colonies (S type) after 18 h, which changed to rough (R type) colonies within 72 h, while the colonies of strain Cd remained smooth and glossy during all the incubation period (up to several weeks).

Changes in the cell surface structure may modify the susceptibility of bacterial cells to adverse environmental factors, xenobiotics in particular. Therefore, we studied the susceptibility of *A. brasilense* Sp7 and Cd to anionic and cationic surfactants. For both strains, minimal inhibiting concentrations (MICs) of SDS comprised 0.02, 0.03, and 0.05% on GSM, MSM, and PM agars, respectively.

Cetavlon MICs were also equal for both strains on MSM and GSM agars and comprised 0.03%. On PM agar, Sp7 growth was inhibited at 0.05%, whereas Cd grew at concentrations twice as high. Both Sp7 and Cd strains formed heavily slimy colonies on PM, presumably, due to changes in the EPS production. It was found earlier that the EPS monosaccharide compositions in strains Cd and Sp7 are different and depend on the carbon source [20]. Thus, the enhanced resistance of strain Cd to cetavlon on PM agar might be explained by differences in levels of production of EPSs and/or in the composition of EPSs.

Changes in the cell surface structure may also have certain effects on cell motility [1–3]. After 18 h of incubation on MSM medium (0.3–0.4% agar), strain Cd formed swarming rings twice as big in diameter as those formed by Sp7. After 36 h of incubation, the disparity in ring diameters was 2.5-fold. In addition, both strains spread faster on media containing less agar than on 0.4% agar. It merits attention that at a Bacto agar concentration of less or equal to 0.35%, *A. brasilense* cells possessed only Fla, whereas at higher agar concentrations, both Fla and Laf were formed [8]. Faster spreading of strain Cd on semisolid media in comparison with Sp7 might result from differences in the structure and/or level of production of cell surface polysaccharides, which decrease the force of friction between cells and medium.

Comparative analysis of the lipopolysaccharides of *A. brasilense* strains Sp7 and Cd. Linear immunoelectrophoresis and polyclonal antibodies to *A. brasilense* Sp7 LPSs allowed determination of LPSI and LPSII characteristic of Sp7 in both Sp7 and Cd strains [17]. However, antibodies to LPSs of strain Cd bound only to LPSI during linear electrophoresis (Fig. 1a). In reactions of double immunodiffusion, LPSI and LPSII in strain Cd were also visualized with antibodies to LPSs of strain Sp7, whereas antibodies to LPSs of strain Cd bound only to Cd LPSI (Fig. 1b). PAAG electrophoresis (Fig. 1c) showed changes in the LPS structure of *A. brasilense* Cd which made it impossible to separate between the two antigens.

Thus, differences in the LPS structure between *A. brasilense* strains Sp7 and Cd are more significant than those between strains Sp7 and Sp7-S [6]. This correlates with the results of probing of the genomes of Sp7, Sp7-S, and Cd (see below). As described earlier by Matora and coauthors [6], LPS preparations from strains Sp7 and Sp7-S contained full-value O-PSs and were identical in the number (two) of antigenic components and PAAG-electrophoresis profiles. According to [6], the change from R type to S type in Sp7 resulted from a change in the contributions of the two O-PSs into the architecture of the cell surface with culture ageing.

As with strains Sp7 and Sp7-S [6], the absorption spectra of the products of the reaction of the LPSs from strains Sp7 and Cd with phenol and sulfuric acid were very similar and had two peaks (Fig. 2). Differences in the height of the peaks at 410–420 nm might result from the structural differences that determine the immunochemical specificity of each LPS. It seems that the immunochemical heterogeneity of LPSI and LPSII in Sp7 [6] and Sp245 [2] is not connected with the principal differences in monosaccharide composition. Yet, the absorption spectra of the products of the reaction of the LPSs from serologically distinct strains Sp7 and Sp245 (Cd and Sp245 as well) with phenol and sulfuric acid had clear differences (Fig. 2).

Comparative physical analysis of DNA of *A. brasilense* strains Sp7 and Cd. Taking into consideration the significant and stable differences in the colony morphology and LPS structure in closely related

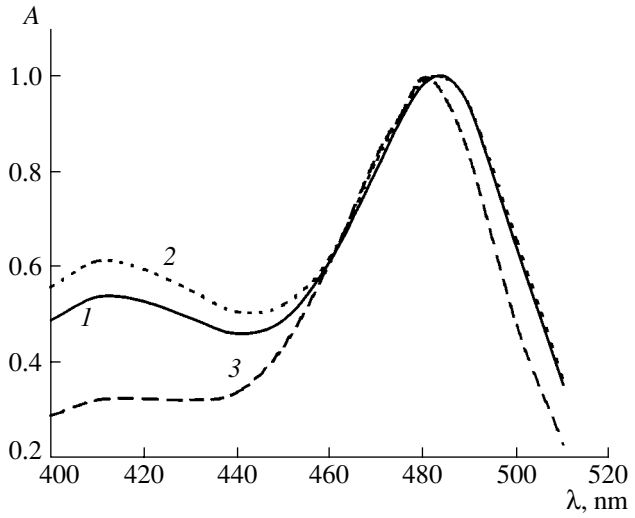

Fig. 2. Absorption spectra of the reaction products with phenol and sulfuric acid of the LPSs of *A. brasilense* (*1*) Sp7, (*2*) Cd, and (*3*) Sp245.

A. brasilense strains Sp7 and Cd, characterized by distinct plant-association backgrounds, the possible genetic cause of these differences is of interest.

In situ cell lysis and gel electrophoresis by the Eckhardt procedure [19] allowed determination in *A. brasilense* Sp7 of 90-MDa and 115-MDa plasmids and several large plasmids with a molecular weight greater than 300 MDa [5]. As in the case of Sp7-S [5], the plasmid profile of *A. brasilense* Cd differed from that of strain Sp7 by the absence of the 115-MDa replicon (Fig. 3a).

Gel electrophoresis of restriction fragments of total DNA from strains Sp7, Sp7-S, and Cd confirmed their close relation (Fig. 3b). As Fig. 3b shows, all the strains are characterized by practically identical *Bam*HI restriction patterns.

For further investigations into genome rearrangements in Sp7-S and Cd, fragments of DNA of plasmids p85 and p120 from *A. brasilense* Sp245 were used as probes (see table).

It should be stressed that the construction of molecular probes on the basis of plasmid DNA of azospirilla is a very complicated process. Azospirilla carry several very large plasmids without any known easily identifiable markers. Therefore, we used cloned fragments of plasmids from strain Sp245 for physical analysis of genetic changes in Sp7 derivatives (see table). Fortunately, this proved to be productive. Probes on the basis of the 120-MDa and 85-MDa plasmids of Sp245 allowed the homology to be revealed between two fragments in p120 and the 90-MDa plasmid and between one fragment in p85 and the 115-MDa plasmid of Sp7 [4].

We found that the patterns of blotting hybridization of the 90-MDa plasmid from Cd and p90 from Sp7 were similar: positive hybridization signals were

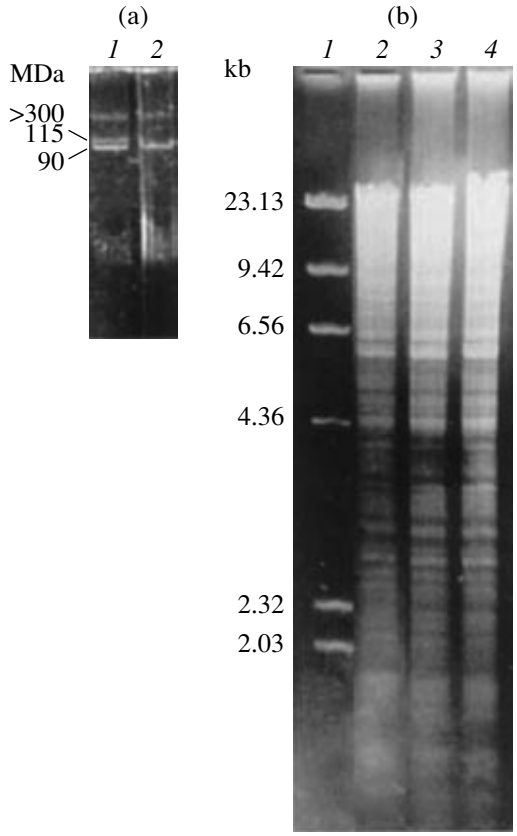

Fig. 3. (a) Gel electrophoresis of plasmid DNA of *A. brasilense* strains (*1*) Sp7 and (*2*)Cd (figures on the left indicate molecular weight of plasmids, MDa). (b) Gel electrophoresis of *Bam*HI-fragments of restriction of total DNA of strains (*2*) Sp7, (*3*) Sp7-S, and (*4*) Cd (figures on the left indicate size of *Hin*dIII-fragments of (*1*) phage λ DNA in kb).

obtained with p120 fragments (Fig. 4c, 4d), and negative with the p85 fragment (Fig. 4b).

A. brasilense strains Sp7 and Cd have similar levels of resistance to ampicillin, which is encoded by p90 [1], up to 500 μg/ml. Strain Cd did not lose its abilities of swimming and swarming, which allows us to conclude that the expression of the *fla* locus and the *laf* locus identified earlier in p90 [1, 3] did not change in a major way. In addition, no differences were revealed by hybridization of *Eco*RI-digestions of the total DNA of Sp7 and Cd with two fragments of p120 carrying the *lps/cal* locus and the *fla/swa* locus and homologous to p90. Thus, we did not find any differences in the p90 functional structure in *A. brasilense* Sp7 and Cd. Blotting hybridization with a 2.4-kb *Eco*RI-generated fragment of p85 carrying the *fla/laf/mot/swa* locus and homologous to p115 revealed only one strong signal instead of two in *Eco*RI digestion of the total DNA of strain Cd, in contrast to strains Sp7, Sp7-S, and Sp245, even under nonstringent conditions (1 M instead of 6 M urea in wash buffer). It was shown that a number of *A. brasilense* genes are duplicated, and homologous copies have different localization, plasmid or chromo-

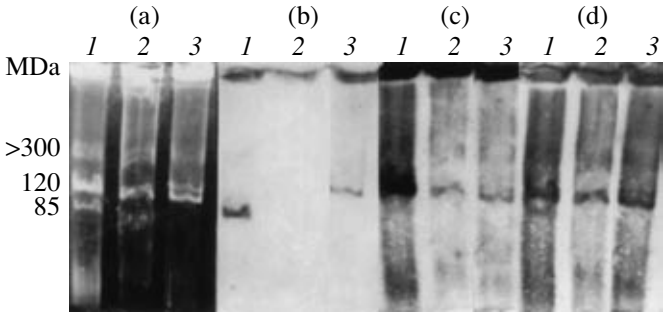

Fig. 4. Homology in plasmid DNA of *A. brasilense* Sp7 and Cd to p120 and p85 fragments from *A. brasilense* Sp245. (a) Plasmids of *A. brasilense* (*1*) Sp245, (*2*) Cd, and (*3*) Sp7 separated by horizontal gel electrophoresis in Tris–acetate buffer and Southern hybridization of this gel with (b) peroxidase-labeled 2.4-kb *Eco*RI-generated fragment of p85 from pEK051X, (c) DNA of p120 from pOmegon-Km-*fla*048X, and (d) DNA of p120 from pOmegon-Km-*lps*348X. Figures on the left indicate molecular weight of plasmids, MDa.

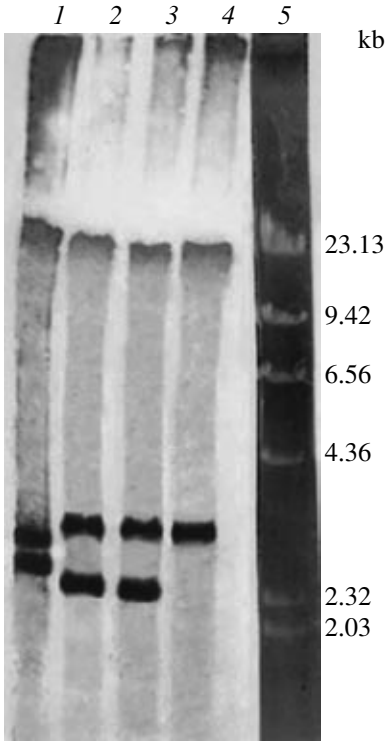

Fig. 5. *Eco*RI-restriction patterns of total DNA of *A. brasilense* strains (*1*) Sp145, (*2*) Sp7, (*3*) Sp7-S, and (*4*) Cd and (*5*) *Hin*dIII-restriction pattern of phage λ DNA. After electrophoresis in agarose gel, fragments of DNA were transferred to Hybond-N^+ membranes by the Southern method and hybridized with peroxidase-labeled probe, which was a 2.4-kb *Eco*RI-fragment of p85 from pEK051X. Figures on the right indicate size of *Hin*dIII-fragments of phage λ DNA in kb.

some [1]. The fragment of p85 we used is also duplicated in *A. brasilense* chromosome [4]. Under stringent conditions, we observed hybridization of this fragment with two *Eco*RI-generated fragments of DNA of Sp245, Sp7, and Sp7-S, and one *Eco*RI-generated fragment of DNA of strain Cd (Fig. 5).

On the basis of our results and other data [7], we may conclude that in strain Sp7-S, at least a certain fragment of p115 or the whole plasmid was integrated into chromosome. This integration is unstable and leads to other plasmid rearrangements, which result in S–R differentiation [7]. On the contrary, in strain Cd, p115 was eliminated, probably in the process of adaptation to another environment [8, 9]. That is why one (2.3 kb) out of two *Eco*RI-generated fragments of total DNA homologous to p85 was not revealed in strain Cd. Another *Eco*RI-generated fragment homologous to p85 is probably localized in chromosome (Fig. 4b, 5). In addition, unlike Sp7-S [7], we did not observe further plasmid rearrangements in Cd. Elimination of p115 or its fragment (significant to regulation of R–S dissociation) resulted in the loss of ability to evolve R phenotype and changes in LPS antigen structure in strain Cd.

Interestingly, the loss of p85, which is related to p115 [1], to p115 and one of two *Eco*RI fragments of total DNA homologous to 2.4-kb *Eco*RI fragment of p85 in *A. brasilense* mutant strain Sp245.5 also resulted in changes of LPS structure [4]. However, the differences in LPS structure of strains Sp245 and Sp245.5 were more drastic than those of strains Sp7 and Cd, presumably due to additional reorganization of the *lps/cal* locus in p120 in Sp245.5 [4].

Probing of genomes of Sp7, Sp7-S, and Cd was carried out with only one relatively small fragment of p85 of strain Sp245 homologous to 115-MDa plasmid of Sp7. Extension of the spectrum of the probes specific to p115 may reveal more significant differences between genomes of strains Sp7 and Cd.

Thus, we found that, in closely related *A. brasilense* strains Sp7 and Cd isolated from different plants [8, 9], stable differences in colony morphology and LPS structure correlated with the loss of 115-MDa plasmid. Genome probing with the fragment of p85 from *A. brasilense* strain Sp245 allowed differentiation between *A. brasilense* strains Sp7 and Cd.

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