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ORIGINAL ARTICLE

Plasmid gene for putative integral membrane protein affects formation of lipopolysaccharide and motility in *Azospirillum brasilense* Sp245

Lilia P. Petrova[1] · Stella S. Yevstigneyeva[1] · Yulia A. Filip'echeva[1] · Andrei V. Shelud'ko[1] · Gennady L. Burygin[1] · Elena I. Katsy[1]

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Abstract

The bacterium *Azospirillum brasilense* can swim and swarm owing to the work of polar and lateral flagella. Its major surface glycopolymers consist of lipopolysaccharides (LPS) and Calcofluor-binding polysaccharides (Cal^+ phenotype). Motility and surface glycopolymers are important for the interactions of plant-associated bacteria with plants. The facultative plant endophyte *A. brasilense* Sp245 produces two antigenically different LPS, LpsI, and LpsII, containing identical O-polysaccharides. Previously, using vector pJFF350 for random Omegon-Km mutagenesis, we constructed a mutant of Sp245 named KM018 that still possessed flagella, although paralyzed. The mutant was no longer able to produce Calcofluor-binding polysaccharides and LpsII. Because of the limited experimental data on the genetic aspects of surface glycopolymer production and flagellar motility in azospirilla, the aim of this study was to identify and examine in more detail the coding sequence of strain Sp245, inactivated in the mutant. We found that pJFF350 was integrated into a coding sequence for a putative integral membrane protein of unknown function (AZOBR_p60025) located in the sixth plasmid of Sp245. To clarify the role of the putative protein, we cloned AZOBR_p60025 in the expression vector pRK415 and used it for the genetic complementation of mutant KM018. The SDS–PAGE, immunodiffusion, and linear immunoelectrophoresis analyses showed that in strain KM018 (pRK415–p60025), the wild-type $LpsI^+$ $LpsII^+$ profile was restored. The complemented mutant had a Cal^+ phenotype and it was capable of swimming and swarming motility. Thus, the AZOBR_p60025-encoded protein significantly affects the composition of the major cell-surface glycopolymers and the single-cell and social motility of azospirilla.

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✉ Elena I. Katsy
ei_katsy@mail.ru

Lilia P. Petrova
petrova_lp@mail.ru

Stella S. Yevstigneyeva
stels20295@yandex.ru

Yulia A. Filip'echeva
ljuche@yandex.ru

Andrei V. Shelud'ko
shel71@yandex.ru

Gennady L. Burygin
burygingl@gmail.com

[1] Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, 13 Prospekt Entuziastov Saratov Russia 410049

Abbreviations

CBPS	Calcofluor-binding polysaccharides
CDS	Coding sequence
CPS	Capsular polysaccharides
EPS	Exopolysaccharides
Km	Kanamycin
LB	Luria–Bertani
LPS	Lipopolysaccharides
MSM	Malate–salt medium
OPS	O-polysaccharide
PDB	Protein Data Bank
Tc	Tetracycline
TSA	Trypton soya agar

Introduction

Azospirilla, best known as plant-growth-promoting bacteria, are well adapted to life in various natural environments and

organisms (Cohen et al. 2004; Baldani et al. 2014). The major surface glycopolymers of these gram-negative bacteria consist of lipopolysaccharides (LPS), capsular polysaccharides (CPS), and exopolysaccharides (EPS). These glycopolymers are essential not only for the maintenance of the structural and functional integrity of azospirilla but also for their interactions with other organisms (e.g., Jofré et al. 2004; Vallejo-Ochoa et al. 2018). In the most studied species of the genus, *A. brasilense*, EPS and CPS form the uncharacterized Calcofluor-binding polysaccharides (CBPS), detectable through bacterial fluorescence on media supplemented with Calcofluor white. Such strains are defined as having Cal^+ phenotype (Del Gallo et al. 1989).

The facultative plant endophyte *A. brasilense* Sp245 (Baldani et al. 1983) is one of the most carefully studied *Azospirillum* strains. Its LPS is represented by the LpsI and LpsII with different antigenic structures and differently charged carbohydrate moieties, the O-polysaccharide (OPS), and/or the core oligosaccharide, which cannot be readily separated from the OPS during its isolation and purification (Katzy et al. 1998; Fedonenko et al. 2004). The OPSs of the LpsI and LpsII are composed of identical pentasaccharide repeating units of D-rhamnose (Fedonenko et al. 2002; Fedonenko et al. 2004).

Like some other *Azospirillum* species, *A. brasilense* has a mixed type of flagellation and can swim and swarm owing to the work of a single constitutive polar flagellum and numerous inducible lateral flagella, respectively (Tarrand et al. 1978; Moens et al. 1995). In strain Sp245, both the lateral and polar flagella are essential for swarming motility (Scheludko et al. 1998). The polar flagellum of this strain is covered with a polysaccharide sheath (Burygin et al. 2007), and the major flagellin of the polar flagellum is supposed to be glycosylated (Filip'echeva et al. 2018). In many other bacteria, flagellins are subject to glycosylation. Such flagellin modifications are often necessary for flagellar assembly and/or work (Merino and Tomás 2014).

The genome of *A. brasilense* Sp245 consists of a chromosome and seven large plasmids (Pothier et al. 2008). The chromosome and six plasmids of Sp245 have been sequenced twice (accession nos. HE577327–HE577333 and CP022253–CP022256 plus CP022260–CP022262). The seventh plasmid of this strain, prone to spontaneous rearrangements (Pothier et al. 2008; Katsy and Prilipov 2009), has been only partially sequenced (accession nos. EU194339; EU595700–EU595706; EU784144; GQ168585; GU904166; GU904167). The predicted coding sequences (CDSs) for motility proteins and for flagellar and glycopolymer production are dispersed between the Sp245 chromosome and several plasmids. Here, we follow the annotation nomenclature for the *A. brasilense* Sp245's plasmids and CDSs that was proposed by the authors of the first genome sequence of this strain (Wisniewski-Dyé et al. 2011; accession nos. HE577327–HE577333) and is used widely in bioinformatics databases.

Previously, we used the suicide plasmid vector pJFF350 (Fellay et al. 1989) for random Omegon-Km mutagenesis of *A. brasilense* Sp245. We isolated six mutants defective in the production of CBPS and LPS: Cal^- $LpsII^-$ KM018, $LpsII^-$ KM139, $LpsI^-$ KM127, KM134, and KM348, and Cal^- $LpsI^-$ KM252 (Katzy et al. 1998). In all the mutants, a single Omegon-Km insertion was located in ~ 120-MDa plasmid (Katzy et al. 1998), later designated as AZOBR_p6 (Wisniewski-Dyé et al. 2011). In the $LpsI^-$ mutant KM348, Omegon-Km inactivated a CDS for a homologue of ADP-heptose:LPS-heptosyltransferase (which performs heptose transfer to the LPS inner core) (Katsy et al. 2010), later called AZOBR_p60094 (Wisniewski-Dyé et al. 2011). In the Cal^- $LpsI^-$ mutant KM252, an Omegon-Km insertion was found in the CDS AZOBR_p60120 for the putative glycosyltransferase homologous to the OPS and CPS biosynthesis enzymes (Katsy and Prilipov 2015).

Of the previously generated Lps mutants of *A. brasilense* Sp245, only KM018 had lost swimming and swarming motility, although its mixed type of flagellation was still the same as in the parent strain Sp245 (Katzy et al. 1998). Because of the limited experimental data on the genetic aspects of surface glycopolymer production and flagellar motility in azospirilla, the aim of this study was to identify and examine in more detail the CDS inactivated in the immotile Cal^- $LpsII^-$ mutant *A. brasilense* KM018.

Materials and methods

Bacterial strains, plasmids, primers, DNA manipulations, and bioinformatics analyses

Strains, plasmids, and primers constructed and/or used in this study are listed in Table 1. All DNA manipulations were done by standard techniques (Sambrook et al. 1989). The presence of *oriV* and the aminoglycoside 3'-phosphotransferase gene (*aphA*) within Omegon-Km made it possible to clone in *E. coli* the AZOBR_p6 DNA, flanking the site of an Omegon-Km insertion in *A. brasilense* KM018. The cloning was done as recommended by Fellay et al. (1989). The self-ligated Km^R *Xho*I fragment of AZOBR_p6 from mutant KM018 (i.e., the plasmid pJFF350-KM018X) was sequenced at Evrogen (Moscow, Russia). Figure 1 shows a scheme for the AZOBR_p6 DNA region that was found altered in KM018.

For complementation of mutant KM018, the CDS AZOBR_p60025 (syn. AZOBR_RS33515; accession no. NC_016597.1 (32421.34064, complement)) of the parent strain Sp245, with its upstream presumed Shine-Dalgarno sequence, was amplified in PCR using iProof high-fidelity DNA polymerase (Bio-Rad, USA) and was cloned into the low-copy-number broad-host-range expression vector pRK415.

Table 1 Bacterial strains, plasmids, and primers

Strain, plasmid, or primer	Relevant characteristics	Reference
Strains		
A. brasilense Sp245 [IBPPM 219][a]	Wild-type strain isolated from wheat roots	Baldani et al. 1983
A. brasilense Sp245 (pRK415)	Sp245 harboring the empty plasmid pRK415; Tc^R	Filip'echeva et al. 2018
A. brasilense KM018 [IBPPM 669][a]	Immotile Cal^- $LpsII^-$ mutant of Sp245 possessing a cointegrate of plasmids AZOBR_p6 (previously known as p120) and pJFF350; Km^R	Katzy et al. 1998
A. brasilense KM018 (pRK415)	KM018 harboring the empty plasmid pRK415; Km^R, Tc^R	This study
A. brasilense KM018 (pRK415–p60025)	KM018 harboring the plasmid pRK415–p60025; Km^R, Tc^R	This study
Escherichia coli DH1	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*; used as a host for pJFF350-KM018X	Sambrook et al. 1989
E. coli DH5α	*supE44* Δ*lac*U169 (ϕ80 *lacZ* ΔM15) *hsdR17 recA1 endA1 gyrA*96 *thi-1 relA1*; used as a host for pRK415 and pRK415–p60025	Sambrook et al. 1989
E. coli K802	*supE hsdR gal metB*; used as a host for pRK2013	Sambrook et al. 1989
Plasmids		
pRK415	RK2-derived broad-host-range, low-copy-number expression vector; 10.7 kb; Tc^R	Keen et al. 1980
pRK2013	Narrow-host-range helper plasmid, repColE1, Tra^+; 48 kb; Km^R	Figurski and Helinski 1979
AZOBR_p6::pJFF350	Cointegrate of plasmid AZOBR_p6 of *A. brasilense* KM018 with vector pJFF350 (i.e., Omegon-Km with $oriV_{pBR322}$ plus *oriT* and defective for transposition IS*1**) (Fellay et al. 1989); Km^R	Katzy et al. 1998
pJFF350-KM018X	Self-ligated *Xho*I fragment of AZOBR_p6::pJFF350 cloned from *A. brasilense* KM018. Contains the 5.3-kb vector pJFF350 and the flanking DNA of AZOBR_p6, including the CDSs AZOBR_p60014–AZOBR_p60025 (without the first 85 bp of AZOBR_p60025); 18.6 kb; Km^R	This study
pRK415–p60025	pRK415 containing the 2069-bp *Hind*III–*Kpn*I DNA fragment with the 1644-bp CDS AZOBR_p60025 from strain Sp245 plus 279 upstream and 146 downstream bp; 12.8 kb; Tc^R	This study
Forward (F) and reverse (R) primers with *Hind*III (H) or *Kpn*I (K) site (italicized) for amplification of AZOBR_p60025		This study
p60025HF	5′-CCCTGTGGCGCTCCC*AAGCTT*TCCTG-3 ′	
p60025KR	5′-CGTTTCCCGTGCT*GGTACC*CTTTTCGTG-3′	

[a] The numbers in square brackets are reference numbers of *A. brasilense* strains in the collection of rhizosphere microorganisms of IBPPM RAS (Saratov, Russia)

The primers (Table 1) were designed to include a *Hind*III and a *Kpn*I site suitable for amplicon cloning in pRK415 downstream of its *lac* promoter. The correctness of the recombinant construct pRK415–p60025 was confirmed by sequencing at Evrogen (Moscow, Russia).

Nucleotide and deduced amino acid sequences and protein structures were retrieved from and/or analyzed on the NCBI (NCBI Resource Coordinators 2014), UniProt (The UniProt Consortium 2015), InterPro (Mitchell et al. 2015), I-TASSER (Roy et al. 2010; Yang et al. 2015), and Protein Data Bank (PDB) (wwPDB Consortium 2019) servers.

Bacterial growth conditions

Azospirilla were grown on a malate–salt medium (MSM) (Döbereiner and Day 1976) containing NH_4Cl (1 g/L), on a

Fig. 1 Scheme for the DNA region of *A. brasilense* Sp245 plasmid AZOBR_p6. The DNA region is altered in the immotile Cal^- $LpsII^-$ mutant *A. brasilense* KM018. In strain Sp245, the CDSs AZOBR_p60024, AZOBR_p60025, and AZOBR_p60026 code for a putative aldolase/epimerase, an integral membrane protein of unknown function, and a protease-like protein (accession nos. CCD03960–CCD03962). The distance between the CDS AZOBR_p60025 and the neighboring CDSs AZOBR_p60024 and AZOBR_p60026 is 167 bp and 306 bp, respectively (accession no. HE577333)

trypton soya agar (TSA) medium or on the Luria–Bertani (LB) medium (Sambrook et al. 1989) at 28 °C. *E. coli* was grown on the LB medium at 37 °C. Solid and soft media contained Bacto Agar at 18 g/L and 5 g/L, respectively. Calcofluor white (Fluorescent Brightener 28, Aldrich) was added to the TSA medium at 1 mg/mL.

Triparental matings of *E. coli* DH5α (pRK415) or *E. coli* DH5α (pRK415–p60025), *E. coli* K802 (pRK2013), and *A. brasilense* KM018 were used to mobilize pRK415 and pRK415–p60025 from *E. coli* to azospirilla (pRK2013 does not replicate in these bacteria). Transconjugants of *A. brasilense* were selected and purified on a solid MSM containing tetracycline (Tc; 25 μg/mL) and kanamycin (Km; 30 μg/mL). Transconjugant *A. brasilense* strains KM018 (pRK415) and KM018 (pRK415–p60025) were always grown in the presence of Km and Tc. The earlier obtained strain Sp245 (pRK415) (Filip'echeva et al. 2018) was grown with Tc. This antibiotic is required for the stable maintenance of pRK415 and its derivatives in bacteria (Keen et al. 1980); thus, in all experiments, all *A. brasilense* strains were grown in the MSM missing Mg^{2+} salts.

Isolation of LPS and their SDS–PAGE analysis

LPS preparations were obtained as described by Yevstigneyeva et al. (2016). Briefly, the bacteria from 48-h broth cultures in the MSM were sedimented by centrifugation. Equal amounts (2 g) of microbial biomass were resuspended in 0.15 M NaCl and agitated on a magnetic stirrer for 6 days with daily replacements of the washing solution with a fresh one. LPS was extracted from the capsule-free microbial biomass with hot 45% aqueous phenol solution by following the modified Westphal method without separation of the water and phenol layers (Kul'shin et al. 1987). Contaminant proteins were precipitated from the LPS solution by addition of 40% trichloroacetic acid to final pH 2.7. The LPS extracts obtained after dialysis were lyophilized. LPS preparations were analyzed by electrophoresis in 15% sodium dodecyl sulfate (SDS)–polyacrylamide gels (PAG) according to Hitchcock and Brown (1983). The gels were stained with silver as described by Tsai and Frasch (1982).

Double immunodiffusion and linear immunoelectrophoresis with anti-LPS polyclonal antibodies

Polyclonal LPS-specific rabbit antibodies, obtained as previously described (Matora et al. 1995), were used at a working concentration of 15 mg/mL. LPS were extracted from the 24-h broth cultures as recommended by Leive et al. (1968). Double immunodiffusion and linear immunoelectrophoresis were done in 1% agarose gels by using the methods of Ouchterlony and Nilsson (1979) and of Krøll (1973), respectively. The gels were stained with Coomassie brilliant blue R250.

Analyses of bacterial motility and flagellation

Bacterial movement in broth and soft agar media was inspected under a Jenaval phase-contrast microscope and was video recorded with a DCR-TRV900E digital camera (Sony, Japan). The swimming rate of 20–40 single cells was calculated as described earlier (Schelud'ko et al. 2009). For assessing bacterial swarming motility, Petri plates with semisolid (0.5% Bacto Agar) media were point inoculated with fresh (42-h) *Azospirillum* cultures from solid media (on solid media, *A. brasilense* forms well-developed colonies on day 2 of incubation). Inoculation of semisolid media was done with an inoculation loop; the initial diameters of the inoculation points were 3–4 mm. The diameters of the swarming rings were determined after static incubation at 28 °C for 24–72 h.

For the analysis of flagellation, bacteria were taken from 24-h broth cultures in MSM and from the colonies grown on semisolid and solid MSM for 48 h. The specimens were applied to formvar-coated grids. After 20 min, the specimens were air dried on a sheet of filter paper, washed with distilled water, dried again, stained with 2% aqueous uranyl acetate for 1–2 min, and examined under a Libra 120 microscope (Carl Zeiss, Germany) at an accelerating voltage of 120 kV. Transmission electron microscopy of the bacteria was done at the Symbiosis Center for the Collective Use of Research Equipment in the Field of Physical–Chemical Biology and Nanobiotechnology at the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences (IBPPM RAS, Saratov, Russia).

Statistical analyses

Quantitative data were obtained from at least three independent experiments in two replicates. Statistical analyses were done with Student's unpaired *t* test; differences were considered significant at $P < 0.05$. Quantitative data are presented as means ± confidence intervals for a 95% significance level. The data were treated by one-way ANOVA. Least significant differences ($LSD_{0.05}$) were determined at a significance level of $P \leq 0.05$.

Results

Identification and analysis of the coding sequence inactivated in *A. brasilense* mutant KM018. Bioinformatics analysis of the predicted translation product of this CDS

We cloned from *A. brasilense* KM018 and sequenced the *Xho*I fragment of AZOBR_p6 containing Omegon-Km. It

turned out that in KM018, the 5.3-kb suicide vector pJFF350 had integrated after the 752nd bp of the 1644-bp CDS AZOBR_p60025 (Fig. 1). The inactive insertion element IS*1** of pJFF350 (Fellay et al. 1989) was found at a long distance from the boundaries between the pJFF350 and AZOBR_p6 DNAs. Only the 4-bp direct repeat of the 5'-CAAT-3' nucleotides was detected in AZOBR_p60025 at the borders with pJFF350.

The CDS AZOBR_p60025 codes for a putative integral membrane protein of unknown function (accession nos. CCD03961 and G8B1A2), which possesses seven predicted transmembrane regions and does not seem to have any kind of signal peptide. For revealing the probable function of this protein, its predicted three-dimensional (3D) structure was generated from its 547-amino-acid-long sequence on the I-TASSER server by using default parameters (Yang et al. 2015). Model 1 of the analyzed protein, which was predicted with a higher confidence, had a C-score of – 1.63 and an estimated TM-score of 0.52 ± 0.15. For the other four predicted 3D models, the correlation between C-score and TM-score was weak, and those models were not used in further work. By using model 1, six closest structural analogues of the CDS AZOBR_p60025-encoded protein were found in PDB. The best hit for the 3D structure of the query protein was the 578-amino-acid-long aminoarabinose transferase ArnT from *Cupriavidus metallidurans* (PDB ID: 5EZM; Petrou et al. 2016). Other relevant structural analogues of the predicted protein product of the CDS AZOBR_p60025 were several membrane oligosaccharyltransferases involved in protein *N*-glycosylation (Table S1).

To examine whether the pleiotropic mutant phenotype of strain KM018 was caused by the inactivation of the CDS AZOBR_p60025, we constructed the recombinant plasmid pRK415–p60025 containing the CDS AZOBR_p60025 of strain Sp245 under the control of the *lac* promoter of pRK415. This promoter is constitutive in *A. brasilense* (Holguin and Glick 2001 and many other studies) that does not utilize lactose (Tarrand et al. 1978).

Restoration of the LPS profile of *A. brasilense* mutant KM018 after its complementation with pRK415–p60025

Comparison of the LPS SDS–PAGE profiles of the five *A. brasilense* strains used in this work revealed a shift in the position of the upper major LPS band in KM018 (pRK415–p60025), as compared with that in strains KM018 and KM018 (pRK415). These data were successfully reproduced by using different clones of the complemented mutant (Fig. 2, lines 5 and 6). The LPS profiles of the complemented clones looked like those of strains Sp245 and Sp245 (pRK415) (Fig. 2).

We also compared the LPS of all the strains under study by double immunodiffusion and linear immunoelectrophoresis

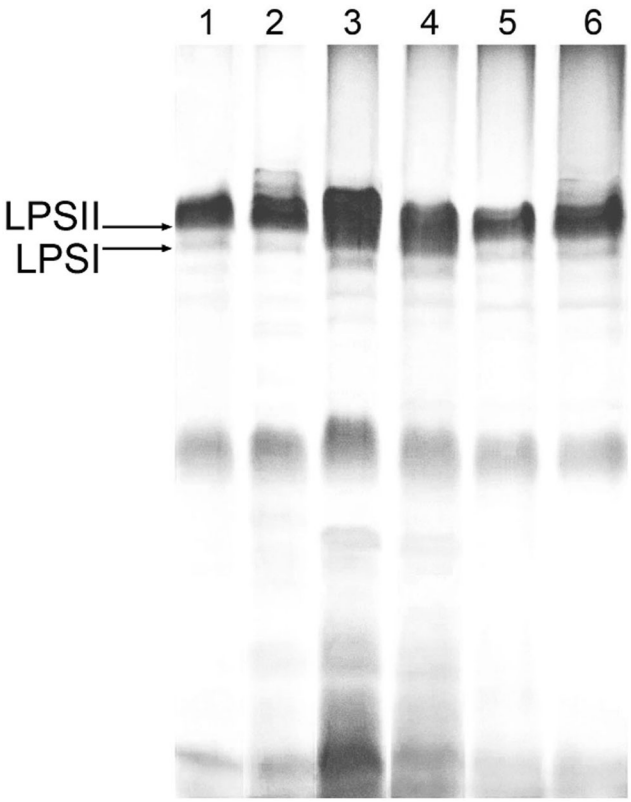

Fig. 2 SDS–PAGE profiles of *A. brasilense* LPS specimens. Strains: (**1**) Sp245; (**2**) Sp245 (pRK415); (**3**) KM018; (**4**) KM018 (pRK415); and (**5**, **6**) KM018 (pRK415–p60025)

(Fig. 3). Rabbit antibodies raised against the LPS of strain Sp245 are known to react with two epitopes. One epitope is shared by both LpsI and LpsII; and the other epitope is present only in LpsII (Katzy et al. 1998). As shown in Fig. 3, the wild-type $LpsI^+$ $LpsII^+$ phenotype was restored in strain KM018 (pRK415–p60025), the LPS of which formed two immunoprecipitation bands with anti-LPS antibodies.

By growing azospirilla on TSA with Calcofluor white, we also checked whether the Cal^+ phenotype was restored in the complemented mutant (Fig. S1). We confirmed that the 3-day colonies of strains Sp245 and Sp245 (pRK415) were already fully fluorescent, while the 3–5 day colonies of strains KM018 and KM018 (pRK415) did not fluoresce under ultraviolet light. The complemented mutant KM018 (pRK415–p60025) gained a Cal^+ phenotype, but its colonies became brightly fluorescent later than those of the wild-type strain—on day 4–5 of incubation.

Effects of the acquisition of pRK415–p60025 on motility of *A. brasilense* KM018 in liquid and soft media

We also examined whether the restoration of the $LpsI^+$ $LpsII^+$ profile in the complemented mutant KM018 was accompanied by any changes in its flagellation and motility. As shown in Fig. S2, all the *A. brasilense* strains under study possessed

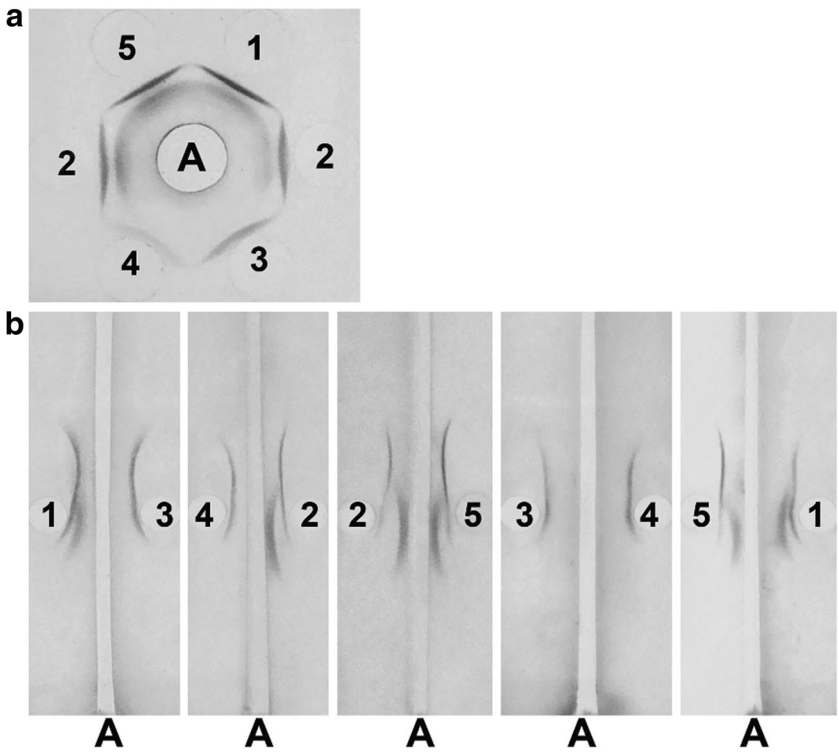

Fig. 3 Double immunodiffusion (**a**) and linear immunoelectrophoresis (**b**) of the LPS extracts from cells of *A. brasilense* with antibodies (A) raised against the LPS of strain Sp245. Strains (1) Sp245, (2) KM018 (pRK415–p60025), (3) KM018, (4) KM018 (pRK415), and (5) Sp245 (pRK415). As found by Katzy et al. (1998), LpsI and LpsII share the same epitope, and LpsII possesses the other epitope

the single polar flagellum in broth media and had a mixed type of flagellation (polar and lateral flagella) on soft and solid media.

In poor (MSM) and rich (LB) liquid media, ~ 80–90 percent of the cells of strains Sp245 and Sp245 (pRK415) were highly motile for the whole period of observation (18–72 h). In broth cultures of strains KM018 and KM018 (pRK415), no motile cells were found. After the acquisition of pRK415–p60025 by mutant KM018, its rare cells were motile in the 18-h cultures, and up to 30 percent of the cells were motile in 48–72 h cultures.

In addition, the complemented mutant was serially passaged in liquid MSM and LB media by transferring 100 μL of the 48-h broth culture to 20 mL of the fresh medium. After three serial passages of KM018 (pRK415–p60025) in liquid media, the proportion of motile cells remained the same as in the initial culture: rare cells were motile in 18-h cultures and up to ~ 30% of the cells were motile after 2–3 days of incubation.

As shown in Fig. 4 and Table 2, strains KM018 and KM018 (pRK415) did not swarm on soft agar media. Strains Sp245 and Sp245 (pRK415) started to form swarming rings from the first day of incubation. In strain KM018 (pRK415–p60025), swarming protuberances appeared on the second day of incubation. Later (on day 3–4), these protuberances transformed into swarming rings with the same diameter as in the wild-type strain. Thus, after a prolonged initial (non-motile) phase of swarming, strain KM018 (pRK415–p60025) started to swarm rather quickly. The complemented mutant did not change its swarming behavior after being isolated from the 72-h swarming protuberances and reinoculated into the fresh soft medium. But in aquatic channels within soft media, motile cells of strains Sp245, Sp245 (pRK415), and KM018 (pRK415–p60025) swam with equal speed (Table 2).

Control strains KM018 and KM018 (pRK415) remained immotile in liquids and on semisolid media and did not form swarming protuberances. Thus, the peculiarities of the swimming and swarming motility of strain KM018 (pRK415–p60025) (Fig. 3; Table 2) do not seem provoked by secondary (suppressor) mutations somewhere in the KM018 genome.

Probably, as in some other swarming bacteria (Wang et al. 2014), as yet uncharacterized biosurfactants (e.g., certain glycopolymers) accumulated on the *A. brasilense* cell exterior and/or in the extracellular milieu. As compared with Sp245 and Sp245 (pRK415), in KM018 (pRK415–p60025), this hypothetical process could occur slower.

Discussion

Plasmid AZOBR_p6 of *A. brasilense* Sp245 (Wisniewski-Dyé et al. 2011; Katsy and Prilipov 2015) and plasmid pRhico of the type strain *A. brasilense* Sp7 (Vanbleu et al. 2004) had been bioinformatically predicted to contain several dozen CDSs involved in carbohydrate metabolism, including polysaccharide biosynthesis. Only a few of those CDSs had been studied in the experiment (Katzy et al. 1998; Katsy et al.

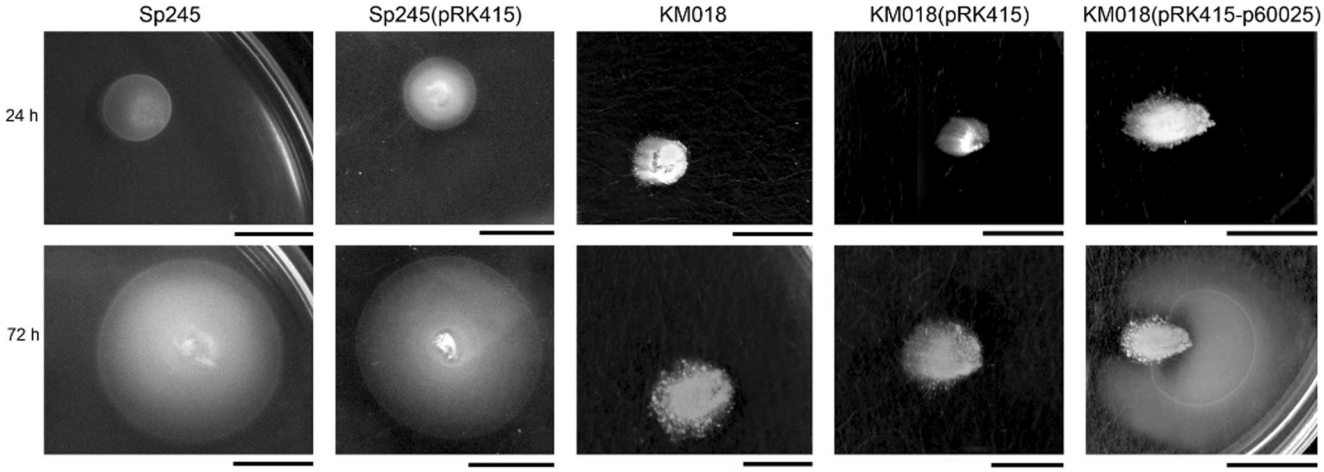

Fig. 4 Behavior of *A. brasilense* strains on the MSM supplemented with 0.5% Bacto agar. Fresh (48-h) bacterial cultures from a solid MSM were stab inoculated in the soft MSM and incubated for 24–72 h. Bars correspond to 1 cm

2010; Katsy and Prilipov 2015; Lerner et al. 2009a, b; Vanbleu et al. 2005).

In this study, we found that in the immotile Cal$^-$ LpsII$^-$ mutant KM018 of *A. brasilense* Sp245, the suicide vector pJFF350 had integrated into the CDS AZOBR_p60025 of the resident plasmid AZOBR_p6. The predicted 3D structure of the deduced translation product of this CDS, an integral membrane protein of unknown function, was analogous to the structures of the glycosyltransferases, which catalyze LPS modification and protein N-glycosylation. The best hit for the 3D structure of the CDS AZOBR_p60025-encoded protein was ArnT, an integral membrane lipid-to-lipid glycosyltransferase. This glycosyltransferase performs the reduction of the negative bacterial membrane charge through the attachment of the cationic sugar 4-amino-4-deoxy-L-arabinose to the phosphate groups of lipid A, which anchors LPS in the outer membrane (Petrou et al. 2016). Two other proteins with similar 3D structures, oligosaccharyltransferases AglB and PglB, transfer a preassembled oligosaccharide from a lipid-linked oligosaccharide donor to acceptor asparagine residues of the proteins (Matsumoto et al. 2013; Napiórkowska et al. 2017). Of note, the LPS biosynthesis and protein N-glycosylation pathways may be evolutionary and functionally related (Hug and Feldman 2011).

Previous analyses of the LPSs isolated from *A. brasilense* Sp245 and its Lps mutants, including KM018, allowed the suggestion that LpsI (with the negatively charged carbohydrate part) was a precursor of LpsII (with the neutral carbohydrate part) (Katzy et al. 1998; Fedonenko et al. 2004). This study was in line with that suggestion. Using the genetic complementation of mutant KM018 and comparative analyses of the SDS–PAGE, immunodiffusion, and linear immunoelectrophoresis profiles of the LPS from the *A. brasilense* strains under study, we showed that the CDS AZOBR_p60025 and its predicted translation product were involved in the formation of LpsII from LpsI. The structural similarity of the CDS AZOBR_p60025-encoded protein of strain Sp245 to the above-mentioned glycosyltransferase ArnT also supports the proposal that it is involved in the modification of LpsI, which leads to the prevalence of LpsII.

Besides the restoration of the wild-type LpsI$^+$ LpsII$^+$ profile in KM018 (pRK415–p60025), the mutant's fluorescence on the agar medium with Calcofluor white was restored. The slower development of such fluorescence (and of the assumed

Table 2 Behavior of *A. brasilense* strains incubated for 3 days on growth media containing 0.5% Bacto Agar

A. brasilense strain	Diameter of swarming rings (mm) formed on soft		Swimming speed (μm/s) of cells in the water channels of soft	
	MSM	LB	MSM	LB
Sp245	22.9 ± 1.4 a	18.2 ± 1.6 a	19.5 ± 1.3 a	19.5 ± 1.3 a
Sp245 (pRK415)	21.8 ± 1.0 a	18.2 ± 2.2 a	19.2 ± 1.2 a	19.2 ± 1.2 a
KM018	ND	ND	ND	ND
KM018 (pRK415)	ND	ND	ND	ND
KM018 (pRK415–p60025)	19.8 ± 3.2 a	18.2 ± 1.7 a	18.8 ± 1.4 a	18.9 ± 1.1 a

Confidence intervals at a 95% significance level are given. In the columns, "a" indicates statistically insignificant differences as determined by one-way ANOVA ($P \leq 0.05$); *ND*, not detected

production of CBPS) than in the wild-type strain may be explained by the changed mode of expression of the CDS AZOBR_p60025 (from plasmid pRK415).

It is known that the state of the cell-surface polymers may considerably affect social behavior of azospirilla. For example, in the presence of the vital dye Congo red (diphenyldiazo-*bis*-α-naphtylamine sulfonate), which can bind to cell-surface glycopolymers and proteins, the swarming of wild-type *A. brasilense* strains is inhibited (Shelud'ko et al. 2006). Although the flagella of the Cal$^-$ LpsII$^-$ mutant KM018 of *A. brasilense* Sp245 were paralyzed, Cal$^-$ LpsI$^-$ (KM252) and LpsII$^-$ (KM139) Omegon-Km mutants were highly motile in liquid and soft media (Katzy et al. 1998). Thus, alterations in the production of CBPS or LpsII *per se* do not seem to affect flagellar motility. However, it cannot be excluded that in mutant KM018, simultaneous defects in the biosynthesis of LpsII and CBPS also negatively affected the structure of the polysaccharide sheath of the polar flagellum and, as a consequence, the polar flagellum-dependent motility. If so, the restoration of the wild-type LPS profile in KM018 (pRK415–p60025) could favor positive changes in the structure of the glycan sheath of the polar flagellum.

The acquisition of the pRK415-borne CDS AZOBR_p60025 by mutant KM018 significantly affected its motility. The observed peculiarities in the swimming and swarming motilities of KM018 (pRK415–p60025) (Fig. 3; Table 2) could be explained by the need for strict control of the expression of the CDS AZOBR_p60025 from its native promoter and by intercellular variability and stochasticity in the expression of the pRK415-borne CDS AZOBR_p60025. In the future, it would be of interest to study the regulation of expression of the CDS AZOBR_p60025 in strain Sp245.

Another possibility is that the CDS AZOBR_p60025-encoded integral membrane protein is involved not only in LpsI modification but also, like its several structural analogues (Table S1), in protein N-glycosylation. The latter process is important for the proper folding and functioning of diverse bacterial proteins (Nothaft and Szymanski 2019). For example, flagellin glycosylation helps to stabilize filament structure and lubricates the rotation of the flagellar bundle (Taguchi et al. 2008).

In the genome of strain Sp245, the CDSs AZOBR_70032, AZOBR_p1160045, AZOBR_p340061, and AZOBR_p410056 (*laf1*) encode putative flagellins with calculated molecular masses of 65.2, 65.2, 28.3, and 43.6 kDa, respectively. Of these putative flagellins, only translation products of the CDSs AZOBR_70032 and AZOBR_p1160045 were detected in the proteomes of the Sp245 cells from broth cultures (Wisniewski-Dyé et al. 2011). Thus, the AZOBR_70032- and AZOBR_p1160045-encoded proteins (accession nos. CCC97396 and CCD01192) are expected to form the filament of the polar flagellum. The 621 amino acid sequences of the predicted 65.2-kDa flagellins of strain Sp245 have 92% identity and 96% similarity. Previously, by using SDS-PAGE and Western immunoblotting of total proteins extracted from the 24-h broth cultures of strain Sp245, the anti-flagellin antibodies were found to recognize two proteins. One protein had an apparent molecular mass of ~ 100 kDa—i.e., a supposed post-translationally modified (most probably, glycosylated) flagellin(s)—and the other had a molecular mass of about 65 kDa—i.e., a predicted primary translation product of the flagellin gene(s) (Filip'echeva et al. 2018). Further chemical analyses are needed for characterizing the structures of the post-translationally modified flagellin(s) of the polar flagellum and its sheath in strains Sp245, KM018, and KM018 (pRK415–p60025).

To conclude, the data obtained show that the CDS AZOBR_p60025-encoded protein is essential for the decoration of the cell surface with LPSII and CBPS and for the single-cell and social motility of *A. brasilense*—i.e., for the traits contributing to the rhizosphere competence of this plant-growth promoting bacterium (Fibach-Paldi et al. 2012).

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S.S. Yevstigneyeva: conduct of research, data analysis.
Yu.A. Filip'echeva: conduct of research, data analysis.
A.V. Shelud'ko: study design, conduct of research, data analysis.
G.L. Burygin: conduct of research, data analysis.
E.I. Katsy: study design and coordination, conduct of research, data analysis, writing of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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References

Baldani VLD, Baldani JI, Döbereiner J (1983) Effects of *Azospirillum* inoculation on root infection and nitrogen incorporation in wheat. Can J Microbiol 29:924–929

Baldani JI, Videira SS, Teixeira KRDS et al (2014) The family *Rhodospirillaceae*. In: Rosenberg E, DeLong EF, Lory S et al (eds) The Prokaryotes: Alphaproteobacteria and Betaproteobacteria. Springer, Berlin, pp 533–618

Burygin GL, Shirokov AA, Shelud'ko AV, Katsy EI, Shchygolev SY, Matora LY (2007) Detection of a sheath on *Azospirillum brasilense* polar flagellum. Microbiology 76:728–734

Cohen MF, Han XY, Mazzola M (2004) Molecular and physiological comparison of *Azospirillum* spp. isolated from *Rhizoctonia solani*

mycelia, wheat rhizosphere, and human skin wounds. Can J Microbiol 50:291–297

Del Gallo M, Negi M, Neyra CA (1989) Calcofluor and lectin-binding exocellular polysaccharides of *Azospirillum brasilense* and *Azospirillum lipoferum*. J Bacteriol 171:3504–3510

Döbereiner J, Day JM (1976) Associative symbiosis in tropical grass: characterization of microorganisms and dinitrogen fixing sites. In: Newton WE, Nijmans CJ (eds) Symposium on nitrogen fixation. Washington State University Press, Pullman, pp 518–538

Fedonenko YP, Zatonsky GV, Konnova SA, Zdorovenko EL, Ignatov VV (2002) Structure of the O-specific polysaccharide of the lipopolysaccharide of *Azospirillum brasilense* Sp245. Carbohydr Res 337:869–872

Fedonenko YP, Zdorovenko EL, Konnova SA, Ignatov VV, Shlyakhtin GV (2004) A comparison of the lipopolysaccharides and O-specific polysaccharides of *Azospirillum brasilense* Sp245 and its Omegon-Km mutants KM018 and KM252. Microbiology 73:143–149

Fellay R, Krisch HM, Prentki P, Frey J (1989) Omegon-Km: a transposable element designed for *in vivo* insertional mutagenesis and cloning of genes in gram-negative bacteria. Gene 76:215–226

Fibach-Paldi S, Burdman S, Okon Y (2012) Key physiological properties contributing to rhizosphere adaptation and plant growth promoting abilities of *Azospirillum brasilense*. FEMS Microbiol Lett 326:99–108

Figurski DH, Helinski DR (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proc Natl Acad Sci USA 76:1648–1652

Filip'echeva YA, Shelud'ko AV, Prilipov AG et al (2018) Plasmid AZOBR_p1-borne *fabG* gene for putative 3-oxoacyl-[acyl-carrier protein] reductase is essential for proper assembly and work of the dual flagellar system in the alphaproteobacterium *Azospirillum brasilense* Sp245. Can J Microbiol 64:107–118

Hitchcock PJ, Brown TM (1983) Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J Bacteriol 154:269–277

Holguin G, Glick BR (2001) Expression of the ACC deaminase gene from *Enterobacter cloacae* UW4 in *Azospirillum brasilense*. Microb Ecol 41:281–288

Hug I, Feldman MF (2011) Analogies and homologies in lipopolysaccharide and glycoprotein biosynthesis in bacteria. Glycobiology 21:138–151

Jofré E, Lagares A, Mori G (2004) Disruption of dTDP-rhamnose biosynthesis modifies lipopolysaccharide core, exopolysaccharide production, and root colonization in *Azospirillum brasilense*. FEMS Microbiol Lett 231:267–275

Katsy EI, Prilipov AG (2009) Mobile elements of an *Azospirillum brasilense* Sp245 85-MDa plasmid involved in replicon fusions. Plasmid 62:22–29

Katsy EI, Prilipov AG (2015) Insertional mutation in the AZOBR_p60120 gene is accompanied by defects in the synthesis of lipopolysaccharide and calcofluor-binding polysaccharides in the bacterium *Azospirillum brasilense* Sp245. Russ J Genet 51:245–250

Katsy EI, Petrova LP, Kulibyakina OV, Prilipov AG (2010) Analysis of *Azospirillum brasilense* plasmid loci coding for (lipo)polysaccharides synthesis enzymes. Microbiology 79:216–222

Katzy EI, Matora LY, Serebrennikova OB, Scheludko AV (1998) Involvement of a 120-MDa plasmid of *Azospirillum brasilense* Sp245 in production of lipopolysaccharides. Plasmid 40:73–83

Keen NT, Tamaki S, Kobayashi D, Trollinger D (1980) Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene 70:191–197

Krøll J (1973) Line immunoelectrophoresis. Scand J Immunol 2:S61–S67

Kul'shin VA, Iakovlev AP, Avaeva SN, Dmitriev BA (1987) Improved method of lipopolysaccharide isolation from gram-negative bacteria. Mol Gen Mikrobiol Virusol 5:44–46

Leive L, Sholvin VK, Mergenhagen SE (1968) Physical, chemical, and immunological properties of lipopolysaccharide released from *Escherichia coli* by ethylenediaminetetraacetate. J Biol Chem 243:6384–6391

Lerner A, Castro-Sowinski S, Valverde A, Lerner H, Dror R, Okon Y, Burdman S (2009a) The *Azospirillum brasilense* Sp7 *noeJ* and *noeL* genes are involved in extracellular polysaccharide biosynthesis. Microbiology 155:4058–4068

Lerner A, Okon Y, Burdman S (2009b) The *wzm* gene located on the pRhico plasmid of *Azospirillum brasilense* Sp7 is involved in lipopolysaccharide synthesis. Microbiology 155:791–804

Matora L, Solovova G, Serebrennikova O, Selivanov N, Shchyogolev S (1995) Immunological properties of *Azospirillum* cell surface: the structure of carbohydrate antigens and evaluation of their involvement in bacteria-plant contact interactions. In: Fendrik I, Del Gallo M, Vanderleyden J, de Zamaroczy M (eds) *Azospirillum* VI and related microorganisms, NATO ASI Series (Series G: Ecological Sciences), v, vol 37. Springer, Berlin, pp 377–382

Matsumoto S, Shimada A, Nyirenda J, Igura M, Kawano Y, Kohda D (2013) Crystal structures of an archaeal oligosaccharyltransferase provide insights into the catalytic cycle of N-linked protein glycosylation. Proc Natl Acad Sci USA 110:17868–17873

Merino S, Tomás JM (2014) Gram-negative flagella glycosylation. Int J Mol Sci 15:2840–2857

Mitchell A, Chan H-Y, Daugherty L et al (2015) The InterPro protein families database: the classification resource after 15 years. Nucleic Acids Res 43:D213–D221

Moens S, Michiels K, Keijers V, van Leuven F, Vanderleyden J (1995) Cloning, sequencing, and phenotypic analysis of *laf1*, encoding the flagellin of the lateral flagella of *Azospirillum brasilense* Sp7. J Bacteriol 177:5419–5426

Napiórkowska M, Boilevin J, Sovdat T, Darbre T, Reymond JL, Aebi M, Locher KP (2017) Molecular basis of lipid-linked oligosaccharide recognition and processing by bacterial oligosaccharyltransferase. Nat Struct Mol Biol 24:1100–1106

NCBI Resource Coordinators (2014) Database resources of the National Center for Biotechnology Information. Nucleic Acids Res 42:D7–D17

Nothaft H, Szymanski CM (2019) New discoveries in bacterial N-glycosylation to expand the synthetic biology toolbox. Curr Opin Chem Biol 53:16–24

Ouchterlony O, Nilsson L-A (1979) Immunodiffusion and immunoelectrophoresis. In: Weir DM (ed) Handbook of Experimental Immunology V 1 Immunochemistry. Alden Press, Oxford, pp 19–33

Petrou VI, Herrera CM, Schultz KM, Clarke OB, Vendome J, Tomasek D, Banerjee S, Rajashankar KR, Belcher Dufrisne M, Kloss B, Kloppmann E, Rost B, Klug CS, Trent MS, Shapiro L, Mancia F (2016) Structures of aminoarabinose transferase ArnT suggest a molecular basis for lipid A glycosylation. Science 351:608–612

Pothier JF, Prigent-Combaret C, Haurat J, Moënne-Loccoz Y, Wisniewski-Dyé F (2008) Duplication of plasmid-borne nitrite reductase gene *nirK* in the wheat-associated plant growth–promoting rhizobacterium *Azospirillum brasilense* Sp245. Mol Plant–Microbe Interact 21:831–842

Roy A, Kucukural A, Zhang Y (2010) I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protoc 5:725–738

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Schelud'ko AV, Makrushin KV, Tugarova AV, Krestinenko VA, Panasenko VI, Antonyuk LP, Katsy EI (2009) Changes in motility

of the rhizobacterium *Azospirillum brasilense* in the presence of plant lectins. Microbiol Res 164:149–156

Scheludko AV, Katsy EI, Ostudin NA et al (1998) Novel classes of *Azospirillum brasilense* mutants with defects in the assembly and functioning of polar and lateral flagella. Mol Gen Mikrobiol Virusol 4:33–37

Shelud'ko AV, Borisov IV, Krestinenko AV et al (2006) Effect of Congo red on the motility of the bacterium *Azospirillum brasilense*. Microbiology 75:48–54

Taguchi F, Shibata S, Suzuki T, Ogawa Y, Aizawa SI, Takeuchi K, Ichinose Y (2008) Effects of glycosylation on swimming ability and flagellar polymorphic transformation in *Pseudomonas syringae* pv. tabaci 6605. J Bacteriol 190:764–768

Tarrand JX, Krieg NE, Döbereiner J (1978) A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Can J Microbiol 24:967–980

The UniProt Consortium (2015) UniProt: a hub for protein information. Nucleic Acids Res 43:D204–D212

Tsai CM, Frasch CE (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal Biochem 119:115–119

Vallejo-Ochoa J, López-Marmolejo M, Hernández-Esquivel AA, Méndez-Gómez M, Suárez-Soria LN, Castro-Mercado E, García-Pineda E (2018) Early plant growth and biochemical responses induced by *Azospirillum brasilense* Sp245 lipopolysaccharides in wheat (*Triticum aestivum* L.) seedlings are attenuated by procyanidin B2. Protoplasma 255:685–694

Vanbleu E, Marchal K, Lambrecht M, Mathys J, Vanderleyden J (2004) Annotation of the pRhico plasmid of *Azospirillum brasilense* reveals its role in determining the outer surface composition. FEMS Microbiol Lett 232:165–172

Vanbleu E, Choudhury BP, Carlson RW, Vanderleyden J (2005) The *nodPQ* genes in *Azospirillum brasilense* Sp7 are involved in sulfation of lipopolysaccharides. Environ Microbiol 7:1769–1774

Wang S, Yu S, Zhang Z, Wei Q, Yan L, Ai G, Liu H, Ma LZ (2014) Coordination of swarming motility, biosurfactant synthesis, and biofilm matrix exopolysaccharide production in *Pseudomonas aeruginosa*. Appl Environ Microbiol 80:6724–6732

Wisniewski-Dyé F, Borziak K, Khalsa-Moyers G, Alexandre G, Sukharnikov LO, Wuichet K, Hurst GB, McDonald WH, Robertson JS, Barbe V, Calteau A, Rouy Z, Mangenot S, Prigent-Combaret C, Normand P, Boyer M, Siguier P, Dessaux Y, Elmerich C, Condemine G, Krishnen G, Kennedy I, Paterson AH, González V, Mavingui P, Zhulin IB (2011) *Azospirillum* genomes reveal transition of bacteria from aquatic to terrestrial environments. PLoS Genet 7:e1002430

wwPDB Consortium (2019) Protein Data Bank: the single global archive for 3D macromolecular structure data. Nucleic Acids Res 47:D520–D528

Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y (2015) The I-TASSER suite: protein structure and function prediction. Nat Meth 12:7–8

Yevstigneyeva SS, Sigida EN, Fedonenko YP, Konnova SA, Ignatov VV (2016) Structural properties of capsular and O-specific polysaccharides of *Azospirillum brasilense* Sp245 under varying cultivation conditions. Microbiology 85:664–671