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EXPERIMENTAL ARTICLES

Effect of the AZOBR_p60123 Plasmid Gene Encoding the Wzt Protein on Lipopolysaccharide Synthesis and Biofilm Formation in the Bacterium *Azospirillum baldaniorum* Sp245

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Abstract—Inactivation of one of the genes (CDS AZOBR_p60123) of the ABC transporter ATP-binding protein Wzt in the p60123::*aphAI* mutant of *Azospirillum baldaniorum* Sp245 and the introduction of an additional copy of this gene into the parent or mutant strain affected the chain-length heterogeneity of O polysaccharides (OPSs) and lipopolysaccharides (LPSs), the OPS and LPS immunochemical characteristics, and the strain properties related to polysaccharide production (biofilm construction). Biofilms of the p60123::*aphAI* mutant Sp245.4-1-1 accumulated two times less biomass than did those of Sp245. Introduction of pRK415-p60123 into the cells of Sp245.4-1-1 or Sp245 resulted in a respective increase or decrease in the ability of the resulting derivative strains to accumulate biofilm biomass, as compared with the original strains.

Keywords: *Azospirillum baldaniorum*, lipopolysaccharides, *wzt* gene, ABC transporters, biofilms

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Bacteria of the genus *Azospirillum* are free-living plant-growth-promoting bacteria (PGPB). So far, 29 *Azospirillum* species have been isolated from different niches in various climatic belts. Most of these species have been isolated from the roots of wild plants, as well as from aquatic environments and polluted areas. They influence the growth and crop yield of numerous plant species, many of them being agronomically and ecologically important, and are widely used as biofertilizers (Hendriksen, 2022). The main surface glycopolymers, i.e., lipopolysaccharides (LPS), capsular polysaccharides (CPS) and exopolysaccharides (EPS), maintain the structural and functional integrity of *Azospirillum* cells, are necessary for biofilm formation and swarming, and provide interactions between bacteria and other organisms (Sheludko et al., 2008, 2018; Zdorovenko et al., 2015; Petrova et al., 2020). In some strains of *Azospirillum baldaniorum* and *Azospirillum brasilense*, EPS and CPS may contain LPS O-polysaccharides (Matora and Shchegolev, 2002; Evstigneeva et al., 2016).

The facultative wheat endophyte *A. baldaniorum* Sp245 (formerly *A. brasilense*) (Dos Santos Ferreira et al., 2020) is one of the most intensely studied *Azospirillum* strains. Its LPS consists of LpsI and LpsII with subtle differences in the structure and charge of the OPS and/or the core, which are detectable by immunochemical reactions and ion exchange chromatography (Katzy et al., 1998; Fedonenko et al., 2002, 2004; Petrova et al., 2020). The repeating unit of OPS as a component of LpsI (negatively charged) and LpsII (uncharged) is a pentasaccharide consisting of D-rhamnose residues (Fedonenko et al., 2002, 2004). The synthesis of homopolymeric OPS, typical of *A. baldaniorum* Sp245 LPS, involves: OPS assembly on a lipid carrier from activated precursors by glycosyltransferases on the cytoplasmic side of the inner membrane; the transfer of OPS to the periplasm by an ATP-binding cassette transporter (ABC-transporter); the ligation of lipid A–core oligosaccharide with OPS and the transfer of LPS to the outer cell membrane (Valvano et al., 2011). The ABC transporters involved in the export of LPS, as well as CPS and EPS, are known to consist of the transmembrane permease Wzm and the ATP-binding protein Wzt (Valvano et al., 2011; Liston et al., 2017). In the genome of strain Sp245, the predicted coding DNA sequences (CDSs) for the enzymes involved in carbohydrate metabolism are scattered throughout the chromosome and several large plasmids. Only a few plasmid genes of *A. baldaniorum* Sp245 and *A. brasilense* Sp7 involved in cell surface polysaccharide production have been analyzed

experimentally (Lerner et al., 2009; Petrova et al., 2020). The AZOBR_p6 Sp245 plasmid is saturated with genes presumably encoding proteins involved in polysaccharide biosynthesis, with many of them probably obtained by horizontal transfer from other bacteria (Wisniewski-Dyé et al., 2011). It contains at least two putative *wzm* (AZOBR_p60016 and AZOBR_p60122) and *wzt* (AZOBR_p60017 and AZOBR_p60123) genes. Previously it has been shown that the genomes of *A. brasilense* strains SR15 and SR75 with homopolymer OPS also contain two putative *wzm* and *wzt* genes each, with very high similarity to the AZOBR_p60016/AZOBR_p60017 and AZOBR_p60122/AZOBR_p60123 genes (Petrova et al., 2017). The genome of *A. brasilense* Sp7 with at least five types of repeating units (the Wzm/Wzt-dependent LPS biosynthetic pathway is not typical of bacteria with heteropolymer OPS) was shown to contain only one of each pair of the putative ABC transporter genes identical to AZOBR_p60017 and AZOBR_p60122 (Petrova et al., 2017).

To confirm the involvement of one of the putative *wzt* genes of *A. baldaniorum* Sp245 in lipopolysaccharide biosynthesis, we inactivated the CDS AZOBR_p60123 and characterized the phenotype of the resultant mutant.

MATERIALS AND METHODS

The strains used were *A. baldaniorum* Sp245 (IBPPM 219), isolated from wheat roots in Brazil (Dos Santos Ferreira et al., 2020), and its derivatives obtained in this work: Sp245(pRK415) and Sp245(pRK415–p60123), the mutant Sp245.4-1-1 (contains an *aphAI* (Km^R cassette) insert from pUC4K in the CDS AZOBR_p60123), Sp245.4-1-1(pRK415), and Sp245.4-1-1(pRK415–p60123). The plasmids and primers used are listed in Table 1. *Escherichia coli* strain DH5α (*supE44* Δ*lac*U169 (ϕ80 *lacZ* ΔM15) *hsdR17 recA1 endA1 gyrA*96 *thi-1 relA1* (Sambrook et al., 1989)) was used to maintain the constructed plasmids. The pRK2013 plasmid was maintained in *E. coli* K802 (*supE hsdR gal metB* (Sambrook et al., 1989)).

Azospirillum strains were grown on a malate–salt medium (Döbereiner and Day, 1976) with NH_4Cl (MSM) at 28°C; *E. coli* was grown on the Luria–Bertani (LB) medium at 37°C (Sambrook et al., 1989). The concentration of Bacto agar in solid media was 20 g/L; the pH values of nutrient media were adjusted to 6.8–7.0. Liquid bacterial cultures were incubated on an Excella E24 shaker incubator platform (New Brunswick Scientific, United States) at 140 rpm. Kanamycin (Km) (30 μg/mL), tetracycline (Tc) (25 μg/mL) and the intravital fluorescent dye Calcofluor (Fluorescent Brightener 28; Sigma-Aldrich, United States) (1 mg/mL) were added as appropriate.

The genomic DNA for PCR was isolated from liquid bacterial cultures by using a GeneJET Genomic DNA Purification Kit (ThermoScientific, United States). The primers (Table 1) and bacterial DNA dissolved in sterile Milli-Q water were added to PCR mixtures to a concentration of 0.5 μM and 0.5–1 μg/50 μL, respectively. The ready-made HS-Taq PCR extra-mix (Diaem) or iProof high-fidelity DNA polymerase (Bio-Rad) were used for DNA amplification.

PCR was performed in a T100 thermocycler (BioRad Laboratories, United States) in at least three independent replicates. PCR products were visualized by electrophoresis in gels containing 1.2–2% agarose under standard conditions (Sambrook et al., 1989). The amplicons and plasmids were purified by using GeneJet PCR Purification and GeneJet Plasmid Miniprep Kits (ThermoScientific, United States), respectively.

DNA hydrolysis with restriction endonucleases, separation of DNA fragments in agarose gel, DNA ligation, and recombinant DNA cloning in *E. coli* cells were performed by conventional methods (Sambrook et al., 1989). Nucleotide sequences were analyzed by using the databases and programs stored on the servers of the United States National Center for Biotechnology Information (NCBI).

For RNA isolation, bacterial cells from the liquid culture were harvested, washed with 50 mM phosphate-buffered saline (PBS, pH 7), and suspended to $OD_{590} = 1$. RNA was isolated from the cells by using NucleoZOL (Macherey-Nagel, Germany), treated with DNase I (NEB) for 30 min at 37°C, and inactivated for 10 min at 65°C. The RNA concentration was determined with Qubit RNA BR Assay Kits on a Qubit 4 fluorimeter (ThermoFS, United States). cDNA was synthesized from 1 μg of RNA by using an MMLV RT kit (Evrogen, Russia) with the addition of the ribonuclease inhibitor Ribolock (ThermoFS, United States). RNA purity was checked by PCR on RNA and cDNA samples by using specific primers (rpoD-118-rt-F and rpoD-118-rt-R) (Table 1) to amplify the housekeeping gene (the *rpoD* gene, encoding the σ^{70} factor; Kumar et al., 2012). The presence of an *RPOD*-specific amplicon in cDNA samples and the absence of any amplicon in RNA samples indicated that the extracted RNA contained no DNA impurities (data not shown). Real-time PCR (RT-PCR) was performed in 25 μL of the mixture with a qPCRmix-HS SYBR + HighROX PCR kit (Evrogen, Russia), by using Applied Biosystems 7300 (Applied Biosystems, United States). For real-time PCR, 4 μL of cDNA and 0.5 μL of each primer (the final concentration of 50 nM) were added to the mixture. The conditions included the initial stage of incubation for 5 min at 95°C, followed by 40 cycles of amplification for 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Specific primers AZOBR_p60123 (primers p60123-rt-F and p60123-rt-R) and AZO-

Table 1. Plasmids and oligonucleotide primers

Plasmid or primer	Characteristic	Source
Plasmids: pRK415	RK2-derived low-copy broad-host-range expression vector, 10.7 kb, Mob^+, Tc^R	(Keen et al., 1980)
pRK2013	Narrow-host-range helper plasmid, repColE1, Tra^+, 48 kb, Km^R	(Figurski and Helinski, 1979)
pJET1.2/blunt	Linearized vector for cloning 6-bp–10-kb PCR products; rep(pMB1), Ap^R, 2974 bp	CloneJET PCR Cloning Kit (Thermo Scientific, United States)
pUC4K	Ap^R Km^R, the source of the *aphAI* gene (Km^R cassette),	(Vieira and Messing, 1982)
pEX18Tc	Tc^R; *oriT*$^+$ sacB$^+$, the plasmid for directed mutagenesis	(Hoang et al., 1998)
pEX18 Tc(p60123::*aphAI*)	pEX18Tc containing an insert of the 556-bp amplicon of the central part of CDS AZOBR_p60123 Sp245, with insertion of the *aphAI* gene, into the amplicon at the BamHI-site; Tc^R Km^R	This work
pRK415-p60123	pRK415 containing the 1371-bp *Pst*I–*Eco*RI DNA fragment with 1224-bp CDS AZOBR_p160123 from Sp245 plus 66 bp upstream and 81 bp downstream of this CDS; Tc^R	
Primers for amplification of Sp245 gene segments:		
p6_160686F	5'-GCTCTGCGCGGCGTGTCCTTTTC-3'	This work
p6_161241R	5'-GCAGCAGCCGGCCCTTCTCCA-3'	
p60123-rt-F	5'-CTGTGACCGGGCTATCCTTC-3'	
p60123-rt-R	5'-GGTTCCGACATCCACGAGTT-3'	
p60017-rt-F	5'-GAGATCGAGCGCAAGATGGA-3'	
p60017-rt-R	5'-CATGGTCGAGCCACAGGATT-3'	
rpoD-118-rt-F	5'-CGTCACCTATGACGAGCTGA-3'	(Kumar et al., 2012)
rpoD-118-rt-R	5'-CTCTTCCGATTCGACGATGT-3'	
Primers* for amplification of CDS AZOBR_p60123 Sp245:		
p160123-PF	5'-CGTG*CTGCAG*CTGTTTTTG-3'	This work
p160123-ER	5'-CGCACC*GAATTC*GATGTAGAA-3'	
Primers to the Km-cassette:		
Km-F	5'-CATCGGGCTTCCCATACA-3'	This work
Km-R	5'-TGCCATTCTCACCGGATT-3'	

* *Pst*I (P) or *Eco*RI (E) restriction sites (in italic) appropriate for the cloning of amplicon in the pRK415 vector were included into the forward (F) and reverse (R) primers. The primers were synthesized by Syntol and Evrogen (Moscow, Russia).

BR_p60017 (primers p60017-rt-F and p60017-rt-R) were used for amplification of the *wzt* genes (Table 1). The *rpoD* housekeeping gene (primers rpoD-118-rt-F and rpoD-118-rt-R; the Table 1) was used as an endogenous control (Kumar et al., 2012). The expression profiles of the target genes were determined relative to the level of expression of the *rpoD* housekeeping gene by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

The procedure of obtaining the mutant included PCR-based production of the central 556-bp segment of the CDS AZOBR_p60123 (by using primers p6_160686F and p6_161241R (Table 1); cloning of this amplicon in the pJET1.2/blunt vector (obtaining of pJET1.2-p60123); cloning by the restriction site of the kanamycin resistance gene from pUC4K (*aphAI*) available in the BamHI amplicon (obtaining of pJET1.2-p60123::*aphAI*); recloning of the resultant

construct from pJET1.2-p60123::*aphAI* into pEX18Tc (obtaining of pEX18Tc-p60123::*aphAI* and transformation of *E. coli* DH5α by this plasmid); use of pEX-18Tc-p60123::*aphAI* for the inactivation and labeling of the CDS AZOBR_p60123 of strain Sp245 with selection and validation of putative double recombinants. The strains *E. coli* DH5α(pEX18Tc-p60123::*aphAI*), *E. coli* K802(pRK2013), and *A. baldaniorum* Sp245 were crossed for this purpose. Kanamycin-resistant but tetracycline-sensitive *Azospirillum* clones were selected (the clones that had undergone double recombination between the pEX18Tc-p60123::*aphAI* plasmid and the target DNA of *Azospirillum*). The insertion of the kanamycin resistance gene into the CDS AZOBR_p60123 of strain Sp245 was confirmed by using primers to the Km cassette from pUC4K (Km-F and Km-R) paired with primers to the AZOBR_p60123 Sp245 gene (Table 1), hybridization of *Azospirillum* colonies and plasmids with the biotin-labeled (Biotin DecaLabel DNA labeling kit, ThermoFS, United States) kanamycin resistance gene, as well as the sequencing of DNA fragments adjacent to the Km resistance gene.

For complementation of the *wzt*::*aphAI* mutation in PCR, an amplicon was generated that contained the 1224-bp CDS of AZOBR_p60123 of strain Sp245 (plus 66 bp upstream and 81 bp downstream of this CDS). For this purpose, forward (p160123-PF) and reverse (p160123-ER) primers (Table 1) were used that contained *Pst*I (P) or *Eco*RI (E) restriction sites suitable for cloning the amplicon in the pRK415 expression vector under the control of its *lac* promoter (this promoter is constitutive in *A. brasilense* and *A. baldaniorum*, which do not utilize lactose; Holguin and Glick, 2001). After the cloning, the pRK415-p60123 plasmid was obtained and transformed in *E. coli* DH5α. The correctness of all constructs obtained was checked by DNA sequencing at Evrogen (Moscow, Russia). Three-parent crosses and the pRK2013 helper plasmid were used to mobilize the pRK415 or pRK415-p60123 plasmids from *E. coli* DH5α cells into *Azospirillum* cells. The transformants that acquired the pRK415 or pRK415-p60123 plasmid were selected and purified on a solid LB medium as described (Petrova et al., 2020).

For LPS isolation, the bacteria were cultivated for 18 h in a liquid MSM. Then the cells were collected and washed from the capsule in PBS (pH 6.8), followed by centrifugation and LPS extraction for 30 min at room temperature in a buffer with pH 8.5 containing 0.1 M Tris-HCl, 10 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% Triton X-100 (EDTA concentration was 0.05 mM per 1 g of wet cells). The cell-free extract was obtained by centrifugation. The EDTA extracts of cells treated with proteinase K were subjected to electrophoresis in 15% polyacrylamide gel in the presence of SDS (SDS–PAGE); the carbohydrate components were visualized by silver staining of the gels (Hitchcock and Brown, 1983). Linear immunoelectrophoresis was performed according to Ouchterlony and Nilsson (1979) by using a Multiphor II device (LKB, Sweden) in 1% agarose gels. The gels were stained with Coomassie brilliant blue R250. Polyclonal rabbit anti-LPS antibodies were obtained as described previously (Matora et al., 1998).

Biofilm formation by *A. baldaniorum* strains was studied in flat-bottomed 96-well polystyrene plates and in glass tubes. Liquid cultures (18 h) were diluted to $OD_{590} = 0.05–0.10$ in LB or MSM. The suspensions were added by 2 mL per tube or 200 μL per plate well and incubated for 6 days under static conditions. The relative amounts of biomass in biofilms were estimated by staining with a 1% aqueous solution of crystal violet and treated as described (Petrova et al., 2017). The biofilm biomass of each strain was determined at least six times. Each time, the biofilms formed in five glass tubes or eight plate wells were stained. The results were processed with Microsoft Office Excel 2010; confidence intervals were determined for the 95% confidence level. One-way analysis of variance (ANOVA) at a significance level of 0.05 was used to find statistically significant differences between the mean values.

RESULSTS AND DISCUSSION

Analysis of the effect of the CDS AZOBR_p60123 on the characteristics of *Azospirillum* surface glycopolymers. Site-directed mutagenesis was done to obtain *A. baldaniorum* mutant Sp245.4-1-1, containing a kanamycin resistance gene insert in the central part of the CDS AZOBR_p60123 of the putative *wzt* gene of the ATP-binding ABC-transporter of (L)PS. The mutant did not differ from the parent strain in the growth rate on solid and in liquid media, flagellation, and cell motility. Similarly to the parent strain, it synthesized polysaccharides binding the intravital dye Calcofluor (Cal^+ phenotype).

The effect of directed mutation in the *wzt* gene on the phenotype of *A. baldaniorum* Sp245 was confirmed by genetic complementation of the *wzt::aphAI* mutant Sp245.4-1-1 by using the obtained pRK415-p60123 construct. The pRK415-p60123 plasmid was also introduced into the cells of strain Sp245 to find a possible effect of increasing the dose of the studied *wzt* gene on its phenotype.

Figure 1a shows the results of SDS–PAGE of the Sp245, Sp245(pRK415–p60123), Sp245.4-1-1(pRK415–p60123), and Sp245.4-1-1 preparations. Compared to strain Sp245 (Fig. 1a, track *1*), mutant Sp245.4-1-1 (Fig. 1a, track *4*) showed a decrease in the number and intensity of bands in the OPS2 region (the zone of localization of this polysaccharide in strain Sp245 was determined by Katzy et al. (1998) by using the appropriate mutants in LPS structure). The electrophoretic profile of the LPS preparations of the complemented mutant Sp245.4-1-1(pRK415–p60123) (Fig. 1a, track *3*), as compared to the

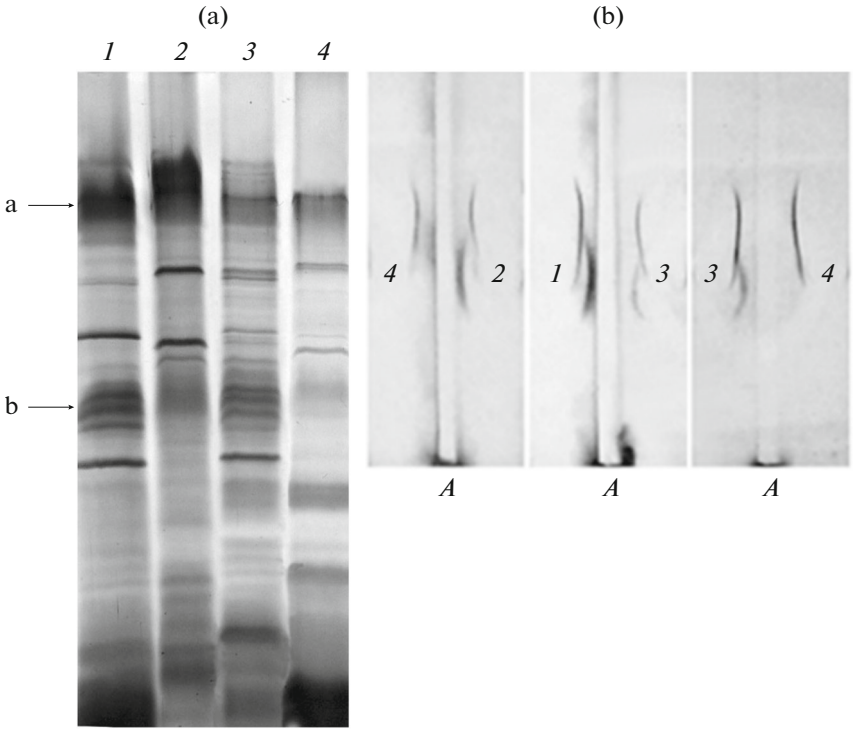

Fig. 1. Characterization of the electrophoretic profiles and immunochemical properties of *A. baldaniorum* preparations: (a) the results of SDS–PAGE of the LPS of strains Sp245 (*1*), Sp245(pRK415–p60123) (*2*), Sp245.4-1-1(pRK415–p60123) (*3*), Sp245.4-1-1 (*4*); (b) the results of linear immunoelectrophoresis of the LPS extracts of strains Sp245 (*1*), Sp245(pRK415–p60123) (*2*), Sp245.4-1-1(pRK415–p60123) (*3*), Sp245.4-1-1 (*4*) with anti-LPS antibodies. Polyclonal rabbit antibodies against the LPS of strain Sp245 were introduced into the central trench (*A*) (as shown previously (Dos Santos Ferreira et al., 2020), LpsI and LpsII contain common epitopes).

Sp245.4-1-1 mutant, had additional bands along the length of the tracks. The bands that appeared in the upper part of zone "a" (the high-molecular-weight fraction of LPS) in strain Sp245.4-1-1(pRK415–p60123) were absent in Sp245.4-1-1 but present in Sp245. Introduction of an additional *wzt* gene copy into parent strain Sp245 also resulted in changes in the electrophoretic profile of the LPS preparations of the resultant Sp245(pRK415–p60123) derivative (Fig. 1a, track *2*). In particular, a greater amount of material was detected in the upper part of zone "a," as compared to the wild type.

As mentioned above, strain Sp245 synthesizes two high-molecular-weight LPS (OPS1 and OPS2) containing an identical D-rhamnan O-polysaccharide but having different charges and antigenic properties (Katzy et al., 1998; Fedonenko et al., 2002, 2004; Petrova et al., 2020). The effect of directed mutation in AZOBR_p60123 on the peculiarities of LPS structure was confirmed by comparative immunochemical analysis of the LPS preparations of all variants under study by using linear immunoelectrophoresis with antibodies against the LPS of the parent strain. In Fig. 1b, one can see that the anti-Sp245 antibodies form two precipitation lines with homologous LPS, which correspond to the negatively charged OPS1 and the neutral OPS2 (Fig. 1b, well *1*), and only one precipitation line with the LPS of mutant Sp245.4-1-1, corresponding to OPS1 (Fig. 1b, well *4*). Yet, the antigenic characteristics of the LPS typical of the parent strain were restored in the complemented mutant Sp245.4-1-1(pRK415–p60123) (Fig. 1b, well *3*). Probably, the inactivation of the CDS AZOBR_p60123 leads to defects in the formation of neutral OPS2, which are eliminated in Sp245.4-1-1(pRK415–p60123) as a result of complementation. Thus, the immunochemical approach provided additional information indicating that the complementary Sp245.4-1-1(pRK415–p60123) synthesizes lipopolysaccharides with heterogeneity not differing from that of the parent strain. It is worth noting that the introduction of the empty vector pRK415 into the bacteria had no effect on the electrophoretic profile and immunochemical characteristics of the LPS preparations of Sp245 (Petrova et al., 2020) or of Sp245.4-1-1.

Analysis of the effect of CDS AZOBR_p60123 inactivation on CDS AZOBR_p60017 expression. The AZOBR_p6 plasmid of *A. baldaniorum* strain Sp245 contains one more copy of the *wzt* gene (CDS AZOBR_p60017). Real-time PCR analysis of the level of transcripts of the second *wzt* gene (CDS AZOBR_p60017) showed that it was 2.5 times higher

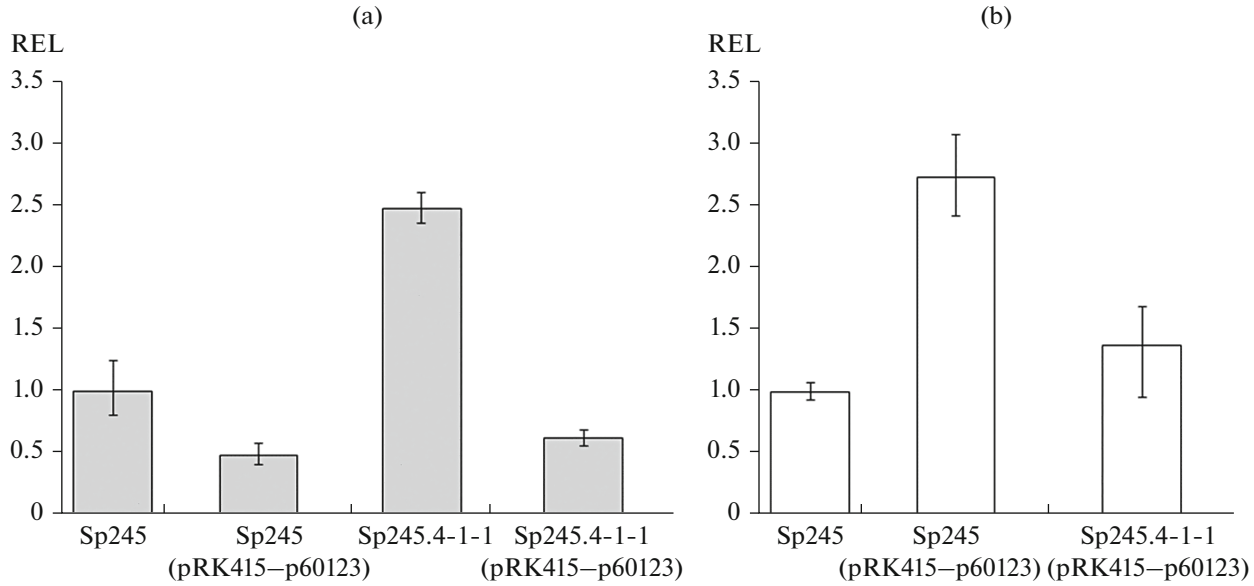

Fig. 2. Results of the expression analysis of the CDS AZOBR_p60017 (a) and the CDS AZOBR_p60123 (b) in *A. baldaniorum* cells. Relative Expression Level (REL) is the ratio of the results of real-time PCR with AZOBR_p60017 and AZOBR_p60123 cDNA from strains Sp245(pRK415–p60123), Sp245.4-1-1, and Sp245.4-1-1(pRK415–p60123) to the analogous results for Sp245.

in the cells of the p60123::*aphAI* mutant Sp245.4-1-1 than the values typical of the parent strain. The introduction of pRK415–p60123 into Sp245.4-1-1 resulted in a significant decrease in CDS AZOBR_p60017 transcription in complemented Sp245.4-1-1(pRK415–p60123) cells to the values 1.7 times below the level typical of Sp245 (Fig. 2a). In the case of the Sp245 derivative with an additional copy of the AZOBR_p60123 gene (Sp245(pRK415–p60123)), CDS AZOBR_p60017 transcription decreased 2.0-fold (Fig. 2a). Thus, the increased expression of the CDS AZOBR_p60017 could partially compensate for CDS AZOBR_p60123 mutation in the p60123::*aphAI* mutant Sp245.4-1-1. Yet, the introduction of the CDS AZOBR_p60123, cloned in pRK415 (pRK415–p60123), into the cells of this mutant or Sp245 leads to a 1.7–2.0-fold decrease in the values characterizing the expression of the CDS AZOBR_p60017 (as compared to the expression level in cells of the parent strain) (Fig. 2).

Analysis of the effect of the CDS AZOBR_p60123 on the accumulation of *Azospirillum* biomass in biofilms. The analysis of biomass accumulation in biofilms has shown that all strains under study did not differ in their ability to form biofilms on glass (Fig. 3). On polystyrene, there was a statistically significant decrease in the relative amount of biomass in biofilms of strain Sp245.4-1-1, as compared to strain Sp245. Yet, the complemented strain Sp245.4-1-1(pRK415–p60123) showed an increase in the relative amount of biomass in biofilms, as compared to mutant Sp245.4-1-1 (Fig. 3). Biofilms of strain Sp245(pRK415–p60123) with an artificially increased dose of the *wzt* gene under study accumulated twice as little biomass as did biofilms of strain Sp245 (Fig. 3). Introduction of the pRK415 plasmid into strains Sp245 and Sp245.4-1-1 had no effect on the quantitative characteristics of biofilms.

LPS have a wide range of OPS chain lengths, which results in a characteristic ladder-like structure when molecules are examined by SDS–PAGE (Holguin and Glick, 2001). The distribution of OPS lengths characteristic of each bacterium is determined by the regulatory mechanisms in the assembly pathways and, in some cases, directly by ABC transporters (Liston et al., 2017). SDS–PAGE of the LPS preparations showed a decreased number and intensity of bands in the area of the OPS2-containing high-molecular-weight fraction for mutant strain Sp245.4-1-1 with the inactivated AZOBR_p60123 gene, as compared to Sp245. Introduction of expression plasmid pRK415–p60123 into the mutant and parent strains resulted in the emergence of additional bands along the length of the tracks in the SDS–PAGE profile of the LPS preparations. Although the complemented mutant Sp245.4-1-1(pRK415–p60123) had no such band intensity as the Sp245 strain, it showed more bands in the zone of shorter OPSs (comparable to the Sp245 LPS profile in this zone). The electrophoretic profile of the LPS of Sp245(pRK415–p60123) demonstrated a significant increase in band intensity and density in the zone of the high-molecular-weight fraction, as compared to Sp245, which apparently was caused by introduction of an additional copy of the *wzt* gene (AZOBR_p60123).

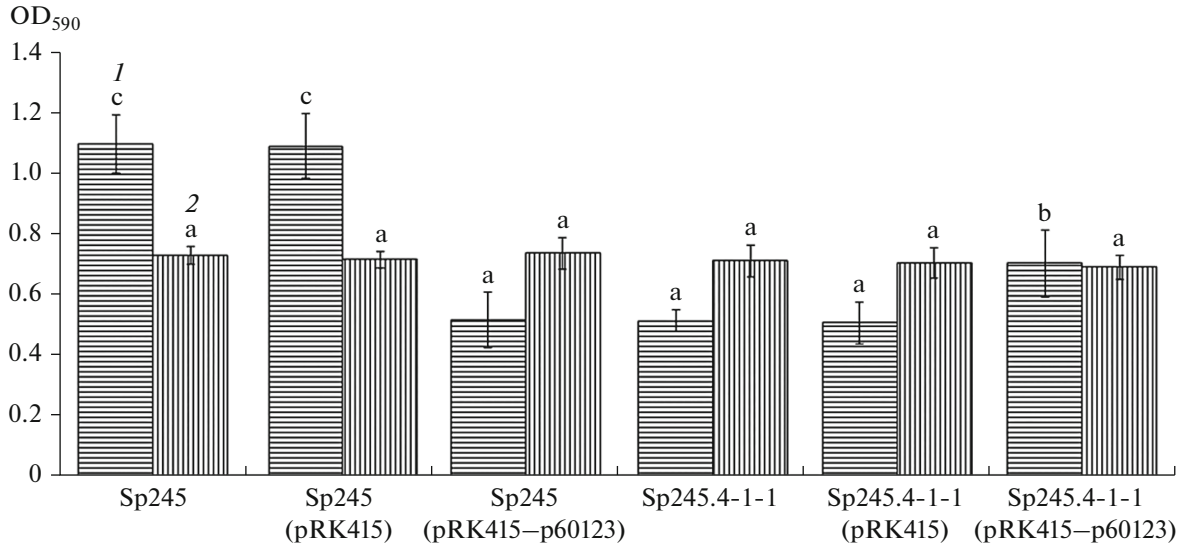

Fig. 3. Relative amount of the biomass in *Azospirillum* biofilms formed over 6 days of cultivation on polystyrene (*1*) or glass (*2*) surfaces under the liquid MSM medium. OD_{590}—desorbed crystal violet after staining of the biofilm biomass.

Nakao et al. (2006) showed that *wzt* gene mutation in the bacterium *Porphyromonas gingivalis* had no effect on the LPS profile. Because *P. gingivalis* has two putative *wzt* genes, the authors assumed the synthesis of an alternative gene product, which compensates for the loss of function of the mutant gene. The AZOBR_p6 plasmid of *A. baldaniorum* Sp245 (Wisniewski-Dyé et al., 2011) has one more copy of the *wzt* gene (CDS AZOBR_p60017), and we observed its increased expression in the mutant, which seems to partially compensate for the mutation in the CDS AZOBR_p60123 in Sp245.4-1-1. The experimental data demonstrate that the introduction of an additional copy of the p60123 gene into Sp245.4-1-1 was accompanied by restoration, though incomplete, of OPS synthesis. At the same time, the immunochemical characteristics of LPS inherent in the parental strain were restored. The low level of CDS AZOBR_p60017 expression, as compared to Sp245 in Sp245.4-1-1(pRK415–p60123) or Sp245 (pRK415–p60123) against the background of the expression of CDS AZOBR_p60123 from the pRK415–p60123 plasmid, probably influenced the level of heterogeneity of OPS chain lengths in these strains, which are determined, inter alia, by ABC transporters (Liston et al., 2017). In the case of Sp245(pRK415–p60123), the level of CDS AZOBR_p60123 expression was elevated, which could also affect the phenotypic characteristics of this strain.

Membrane LPS and the excreted polysaccharides (CPS and EPS) of bacteria, including those of *Azospirillum*, fix mature biofilms on solid surfaces, stabilize biofilm biomass by performing the frame function, and are necessary for the collective motion of bacteria (Sheludko et al., 2018; Petrova et al., 2020). The impaired synthesis of polysaccharides often leads to changes in biofilm formation and motility of swarming *Azospirillum* (Sheludko et al., 2007; Petrova et al., 2020). The results of this study showed that the biofilms of mutant Sp245.4-1-1 accumulated half as much biomass as did those of strain Sp245 on the hydrophobic surface. The introduction of pRK415–p60123 into Sp245.4-1-1 or Sp245 cells increased or decreased the ability to accumulate biomass in biofilms by the resultant derivatives, as compared to the original strains, respectively. Because all the studied strains did not differ in their growth rate in liquid planktonic cultures and on solid media and formed biofilms with the same amount of biomass on the hydrophilic surface, we can assume that cell division is not the parameter limiting biomass accumulation in biofilms.

Preservation of the polar flagellum (Fla) by Sp245 cells integrated into a mature biofilm contributes to the maintenance of biofilm integrity and increases biofilm stability under hydrodynamic shear conditions (Sheludko et al., 2015). The cells of the strains under study synthesized the polar flagellum and swam in the liquid medium at the same speed. LPS may promote additional inclusion of motile bacteria from the planktonic culture into the biofilm; however, all strains formed similar biofilms on the hydrophilic surface but different films on the hydrophobic surface. Thus, additional inclusion of planktonic bacteria into the already formed biofilm hardly plays a significant role; probably, the differences begin to manifest themselves at the early stages of film formation. At the initial stages of biofilm formation, motile bacteria formed thin films, in which microscopy revealed scattered cell aggregates easily washed off the surface during the aspiration of suspensions and washing with water. At this stage, the cells of strains Sp245, Sp245(pRK415–

p60123), Sp245.4-1-1, and Sp245.4-1-1(pRK415–p60123) with different LPS heterogeneity can differently interact with the colonized surface and with each other. LPS heterogeneity, which is determined, among other things, by the molecular weights of O-polysaccharides, correlates with the forces of adhesion and adherence at the levels of both individual bacterium and bacterial cell population (Jucker et al., 1997; Camesano and Abu-Lail, 2002; Atabek and Camesano, 2007). OPS chain length mediates the hydrophobicity and charge of bacterial cells and polysaccharides per se (Atabek and Camesano, 2007). These parameters were obviously individual for cells of each of the strains. The bacteria moved differently at the liquid medium/solid surface interface, interacted with each other or with the colonized surface (hydrophobic or hydrophilic), which probably influenced the following stages of biofilm formation: strong bacterial adhesion on the surface, the beginning of biomass growth, and biomass stabilization, typical of *A. baldaniorum* Sp245 (Sheludko et al., 2015).

It can be assumed that the phenotypic differences (immunochemical characteristics and changes in biofilm formation) of *A. baldaniorum* Sp245, Sp245(pRK415–p60123), Sp245.4-1-1, and Sp245.4-1-1(pRK415–p60123) are due to the LPS heterogeneity of these strains, which is mediated by the activity of the *wzt* gene (AZOBR_p60123).

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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