Spontaneous Super-Swarming Derivatives of *Azospirillum brasilense* Sp245 have Different DNA Profiles and Behavior in the Presence of Various Nitrogen Sources

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Abstract *Azospirillum brasilense* swims in liquid environments and swarms in semisolid media. Five variants of *A. brasilense* Sp245, Sp245.P1–Sp245.P5, which swarmed faster than Sp245 in a semisolid malate–salt medium, have been isolated. In Sp245.P1–Sp245.P4, a new megaplasmid was revealed instead of an indigenous 85-MDa plasmid (p85). By polymerase chain reactions (PCR) with primers to the segments of p85 important for proper bacterial motility/flagellation and for dissimilatory nitrite and NO reduction, that DNA of p85 was found retained by all the variants. In ERIC- and RAPD-PCR, microdiversity between the total DNAs of Sp245 and its variants was detected. Interstrain differences in growth characteristics in liquid peptone–succinate–salt medium with KNO₃ or KNO₂ and in KNO₂ production/consumption were revealed. Although all the variants swam and swarmed faster than Sp245 in the medium supplemented with NH₄Cl or KNO₃, not all of them could do so in MPSS with KNO₂.

Keywords *Azospirillum* · Plasmid plasticity · Motility · Nitrate reduction

Introduction

The plant-growth-promoting rhizobacteria *Azospirillum brasilense* exhibit different motility mechanisms potentially important for the formation of the associations with

plants [2]. They swim in liquid environments by rotating a single polar flagellum and swarm on semisolid surfaces, which depends on numerous inducible lateral flagella and on intercellular interactions [2, 7, 20].

Several spontaneous derivatives of *A. brasilense* Sp245 (named Sp245.P1, Sp245.P2, Sp245.P3, Sp245.P4, and Sp245.P5) that swarm faster than the parent strain were isolated and partially characterized [19]. The diameter of the swarming rings formed by Sp245.P1–Sp245.P5 in semisolid malate–salt medium (MSM) exceeded that in Sp245 approximately twofold. The mode of bacterial flagellation in liquid environments and on surfaces, colony morphology on solid MSM, and growth rates in broth MSM were similar in *A. brasilense* Sp245 and its super-swarming variants [19]. Recently, it was shown that the swarming motility of *A. brasilense* Sp245 varies significantly depending on the nitrogen source. In media with nitrate or nitrite, a decrease in the diameter of the swarming rings formed by *A. brasilense* Sp245 was observed, compared to bacteria grown in the presence of ammonium or molecular nitrogen [20].

The purpose of this work was to analyze in more detail the super-swarming variants of *A. brasilense* Sp245 and to compare their growth and motility in the presence of different N sources.

Materials and Methods

Bacterial Strains and Growth Conditions

This study used *A. brasilense* strain Sp245 [1] and previously isolated spontaneous super-swarming derivatives Sp245.P1–Sp245.P5 [19], which have been deposited in the culture collection of the Institute of Biochemistry and

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Bacteria were grown at 30 °C on medium with peptone, succinate, and salts (MPSS; pH 6.8) [6] supplemented with NH_4Cl , KNO_3 , or KNO_2 , 1 g l^{-1} . Solid and semisolid media contained Bacto agar at 18 and 4 g l^{-1} , respectively. Broth cultures were incubated under aerobic conditions in a rotary shaker incubator (New Brunswick Scientific, USA) (140 rpm) or without agitation; bacterial growth was checked by the absorbance at 590 nm (A_{590}). The inoculated solid and semisolid media were incubated in a thermostat for 36–72 h.

The morphology of the zones of bacterial migration from the inoculation point into semisolid MPSS was examined by eye and by phase contrast microscopy. Bacterial swimming speed was determined as described by Shelud'ko et al. [19].

Checking Nitrate/Nitrite Reduction

Bacterial nitrate and nitrite reduction were detected by using a diazotization-based colorimetric assay, with zinc powder, as required [13]. Nitrite concentration in centrifuged (cell-free) cultural media was measured by the absorbance of the reaction mixtures at 540 nm (A_{540}).

DNA Analyses

Plasmid profiles were analyzed by the Eckhardt procedure [4]. For PCR fingerprinting of the *A. brasilense* genomes, we used primers specific to repetitive BOX elements [18] and to enterobacterial repetitive intergenic consensus (ERIC) and repetitive extragenic palindromic (REP) sequences [3], as well as primers for random amplified polymorphic DNA (RAPD) analysis [5] (Table 1).

Previously, it was found that alterations in the primary structure of an 85-MDa plasmid of *A. brasilense* Sp245, p85, provoked by its cointegration with a vector for Omegon-Km mutagenesis, pJFF350, could result either in severe defects in bacterial flagellation and/or motility or in the acceleration of swimming and swarming [9]. Apart from the open reading frames (*orf*) whose predicted products could affect flagellar structure or rotation (*ccoN*, *orf414*, *orf418*, *orf319*, and *orf119*) [10], several *orf* with proposed roles in nitrite and NO reduction (*nirK* and *norCBQD*), NO sensing (*orf181*), and NO detoxification (*metC*) [16] were found in p85. For comparing the DNAs of *A. brasilense* Sp245 and its super-swarming derivatives, we used six pairs of primers (P) specific to several loci of the reported nucleotide sequences of an 85-MDa plasmid (p85) of *A. brasilense* Sp245 (Table 1).

Table 1 Primers used

Primer (pair) and PCR target locus	Oligonucleotide	Position in the p85 GenBank sequence (where appropriate)	GenBank accession number and/or reference
Primers for REP-PCR			
BOX	5'-CTACGGCAAGGCGACGCTGACG-3'		[18]
ERIC	5'-ATGTAAGCTCCTGGGGATTCAC-3'		[3]
	5'-AAGTAAGTGACTGGGGTGAGCG-3'		
REP	5'-IIIIICGICGICATCIGGC-3'		[3]
	5'-ICGICTTATCIGGCCTAC-3'		
RAPD	5'-GTTTCCGCCC-3'		[5]
p85-Specific primers			
P1, <i>nirK</i>	5'-CGGCGCCGTGGGTGGTCT-3'	4938–4955	EU194339, [21]
	5'-CGGTGGCAAGCGGGAAATG-3'	5403–5385	
P2, <i>orf293</i>	5'-CGTCGGCGCCTTGTTGAGAAT-3'	7042–7062	EU194339, [21]
	5'-CGGTGCGCCTGCTGCTGGAAGA-3'	7452–7431	
P3, <i>orf293/ccoN</i>	5'-CCGGATGCGCGTGCTGAC-3'	7805–7822	EU194339, [21]
	5'-CGGCCCCGGGTATGGAT-3'	8226–8210	
P4, <i>ccoN/metC</i>	5'-TGTGGGTGTGCGGCATCAT-3'	9439–9457	EU194339, [21]
	5'-GAATCGGCGGCGTCACC-3'	9847–9831	
P5, <i>orf414</i>	5'-CGACGGGGAGGCGCTGGTGGACT-3'	1210–1232	EU595706, [21]
	5'-CGGCACGGTGATGGAAGACG-3'	1645–1626	
P6, <i>orf319</i>	5'-CGCGGCGCCGGAAGTCAG-3'	170–187	GQ168585, [10]
	5'-CGGGTCGCGGTGGATGTGGAT-3'	699–679	

For PCR, fresh bacterial cultures were suspended in 20 μ l of a freshly prepared lysis buffer (50 mM NaOH, 0.25 % SDS) and were boiled for 15 min. The lysate was diluted with 200 μ l of MILLI-Q water and centrifuged for 5 min. Four μ l of the supernatant supplemented with primers, 25 pM each; four dNTP (Fermentas, Lithuania), 0.2 mM each; and 1.25 U *Taq*-polymerase (Fermentas) in the final volume of 50 μ l were used for PCR. The amplification conditions with P1–P10 were as follows: 10 min at 95 °C, *Taq*-polymerase addition; 25 cycles with the following temperature profile: denaturation at 94 °C for 30 s; annealing at 67.3 °C (for P1 and P2), 66.9 °C (P3), 67.0 °C (P4), 69.3 °C (P5), or 66.8 °C (P6) for 1 min; and extension at 70 °C for 4 min. After 25 cycles, the reaction mixtures were incubated for an additional 5 min at 70 °C. The amplification conditions for BOX-, ERIC-, RAPD-, and REP-PCR were as described earlier [3, 5, 18]. A Tercyc automated thermocycler (DNA-Technology, Russia) was used for DNA amplification. PCR products were analyzed by gel electrophoresis in 2 % agarose.

All the experiments were repeated at least three times. Quantitative data were treated statistically with the program Microsoft Office Excel 2003. The confidence intervals are given for a 95 % significance level.

Results and Discussion

Comparative Analysis of Plasmid and Total DNAs of the *A. brasilense* Wild-Type and Super-Swarming Strains

Various *A. brasilense* strains were found to possess multipartite genomes composed of one or more chromosomes

and several large plasmids [11]. In *A. brasilense* Sp245, plasmids with molecular weights of 85, 120, and more than 300 MDa could be revealed [8]. Unexpectedly, a novel, high-molecular-weight plasmid was found in four of the super-swarming variants of Sp245 (Sp245.P1–Sp245.P4) instead of p85 (e.g., Fig. 1c). The variant Sp245.P5 had the same plasmid profile as Sp245 (Fig. 1c).

Several studies have recently shown that *Azospirillum* genomes are quite dynamic and that some plasmids are involved in genomic rearrangements [8, 14, 15, 17, 21, 23]. For example, Pothier et al. [17] noticed differences in the plasmid contents of several *A. brasilense* Sp245 stocks kept in different laboratories. Those authors supposed that the smallest plasmid found in the Sp245 strains from Rehovot (Israel) and Leuven (Belgium) had formed a cointegrate with another replicon in the Sp245 strain from Lyon (France) and had been lost by the strain used in the Sp245 genome sequencing project in Knoxville (USA) [17]. In this work, the plasmid profiles of *A. brasilense* Sp245 and Sp245.P5 were similar to those of the Rehovot and Leuven Sp245 strains, and the novel plasmid of Sp245.P1–Sp245.P4 looked like the probable plasmid cointegrate from the Lyon strain (Fig. 1c).

Using PCR with p85 targeted primers (Table 1), we checked whether the corresponding loci of p85 were still present in the spontaneous super-swarming variants of Sp245. All the PCR on the DNAs isolated from the six *A. brasilense* strains gave identical positive results (e.g., Fig. 2). These data strongly suggested that the novel plasmid found in Sp245.P1–Sp245.P4 (Fig. 1c) was a derivative of p85.

In BOX- and REP-PCR, we also found no differences between the DNAs of Sp245 and its super-swarming variants. Possibly, the corresponding DNA repeats were

Fig. 1 Results of the analyses of the *A. brasilense* DNAs. Amplification products of ERIC- (a) and RAPD- (b) PCR on the DNAs of Sp245 (1), Sp245.P1 (2), and Sp245.P2 (3). (No differences were revealed between the DNAs of Sp245 and Sp245.P3–Sp245.P5.)

M marker of molecular size, 100 bp+DNA ladder (Fermentas, Lithuania). Plasmid profiles (c) of Sp245 (1), Sp245.P1 (=Sp245.P2, Sp245.P3, and Sp245.P4) (2), and Sp245.P5 (3). Arrows indicate the differences between the strains

Fig. 2 Results of PCR on the total DNAs of *A. brasilense* Sp245 (1) and Sp245.P1 (2) (=Sp245.P2, Sp245.P3, Sp245.P4, and Sp245.P5) by using primers P1–P6. M: 100 bp+DNA ladder (Fermentas, Lithuania)

not involved in/affected by genomic rearrangements. However, in ERIC- and RAPD-PCR, we detected microdiversity between the DNAs of Sp245 and its variants Sp245.P1 and Sp245.P2 (Fig. 1a, b).

Growth and Motility of the *A. brasilense* Wild-Type and Super-Swarming Strains in the Presence of Different Nitrogen Sources

It has previously been shown that the growth rates of *A. brasilense* Sp245 and its super-swarming variants in MSM with NH_4Cl were practically identical [19]. In this work, we compared the growth of the six *A. brasilense* strains in MPSS supplemented with either KNO_3 or KNO_2 under conditions of intensive aeration (e.g., Fig. 3). No differences were found between the growth curves for *A. brasilense* Sp245 and its super-swarming variants Sp245.P1, Sp245.P2, and Sp245.P4 (data not shown). However, *A. brasilense* Sp245.P5 reached significantly higher final cell densities in the presence of KNO_3 or KNO_2 as compared to the other strains, and *A. brasilense* Sp245.P3 exhibited a more prolonged lag phase during growth in MPSS with KNO_2 than the other strains (Fig. 3).

After 16 h of incubation, when the interstrain differences in growth in MPSS with KNO_3 became visible, we started to monitor the concentration of KNO_2 in the cultural media. As shown in Fig. 4, *A. brasilense* Sp245.P3 differed from Sp245 and Sp245.P5 in that it showed the capacity for an almost complete transformation of KNO_2 . Nitrite produced by azospirilla in liquid MPSS with KNO_3 could be the product of assimilatory or dissimilatory nitrate reduction. Although, as an alternative form of respiration, denitrification mainly occurs when O_2 concentrations are limiting, various bacterial species, including *A. brasilense*, are also capable of aerobic dissimilatory reduction of N oxides [12, 22].

Fig. 3 Growth of *A. brasilense* Sp245 and its super-swarming variants Sp245.P3 and Sp245.P5 in liquid MPSS under aerobic conditions. (The growth curves for *A. brasilense* Sp245.P1, Sp245.P2, and Sp245.P4 looked like that for Sp245)

Fig. 4 Nitrite production and consumption during aerobic growth of *A. brasilense* in liquid MPSS with KNO_3

Under oxygen limitation, when the *A. brasilense* cultures were incubated in liquid MPSS with KNO_2 without agitation, they grew equally slowly, and after 52 h incubation they reached final densities of only approximately 0.4 to 0.5 at A_{590} . In MPSS with KNO_3 , the density of a 52-h-old *A. brasilense* Sp245.P5 stationary culture was approximately twofold higher (~ 1.0 at A_{590}) than that in all the other strains (data not shown).

In liquid MPSS with NH_4Cl or KNO_3 , cells of the *A. brasilense* super-swarming variants had significantly higher swimming speeds than the Sp245 cells. In liquid MPSS with KNO_2 , *A. brasilense* Sp245 and Sp245.P3 swam slower (the swimming speed of Sp245.P3 was

Table 2 Swimming and swarming of *A. brasilense* Sp245 and its variants in the presence of different nitrogen sources

<i>A. brasilense</i> strain	Swimming speed ($\mu\text{m s}^{-1}$) of the cells in 18-h-old cultures in liquid MPSS with			Swimming speed ($\mu\text{m s}^{-1}$) of the cells in 36-h-old swarms on semisolid MPSS with			Diameter of the swarming rings (mm) formed in 36 h in semisolid MPSS with		
	NH ₄ Cl	KNO ₃	KNO ₂	NH ₄ Cl	KNO ₃	KNO ₂	NH ₄ Cl	KNO ₃	KNO ₂
Sp245	28.9 $\pm$ 1.1	29.0 $\pm$ 1.5	24.5 $\pm$ 2.0	15.8 $\pm$ 0.9	15.5 $\pm$ 1.0	13.5 $\pm$ 0.8	17.4 $\pm$ 2.8	12.3 $\pm$ 1.0	7.7 $\pm$ 1.0
Sp245.P1	36.6 $\pm$ 2.1	33.8 $\pm$ 1.0	31.4 $\pm$ 1.5	19.1 $\pm$ 0.9	18.2 $\pm$ 1.2	14.5 $\pm$ 1.3	35.8 $\pm$ 2.0	17.2 $\pm$ 2.4	8.6 $\pm$ 1.4
Sp245.P2	34.2 $\pm$ 1.8	ND	ND	ND	ND	ND	35.0 $\pm$ 2.1	16.5 $\pm$ 2.1	8.2 $\pm$ 1.4
Sp245.P3	35.9 $\pm$ 2.1	34.2 $\pm$ 1.4	26.8 $\pm$ 2.3	19.2 $\pm$ 1.0	18.7 $\pm$ 1.5	14.0 $\pm$ 1.2	36.2 $\pm$ 1.7	15.0 $\pm$ 0.8	6.1 $\pm$ 0.5
Sp245.P4	36.6 $\pm$ 3.3	ND	ND	ND	ND	ND	33.1 $\pm$ 2.3	16.1 $\pm$ 2.0	7.1 $\pm$ 1.1
Sp245.P5	36.0 $\pm$ 2.1	34.9 $\pm$ 1.5	32.7 $\pm$ 3.0	19.9 $\pm$ 1.0	19.1 $\pm$ 1.2	18.4 $\pm$ 1.2	37.9 $\pm$ 1.4	24.7 $\pm$ 1.9	17.1 $\pm$ 2.7

ND not determined

suppressed to the wild-type level), but Sp245.P1 and Sp245.P5 swam almost as quickly as in the other two media (Table 2). After 20–22 h incubation in MPSS with nitrate, the cultural media of *A. brasilense* Sp245 and its variants Sp245.P1, Sp245.P2, Sp245.P3, Sp245.P4, and Sp245.P5 contained KNO₂ at almost equal concentrations of 25.0 ± 4.8 , 23.8 ± 3.4 , 24.0 ± 4.6 , 26.7 ± 5.2 , 23.7 ± 4.7 , and $22.6 \pm 2.5 \text{ mg l}^{-1}$, respectively, and such (or even approximately twofold higher ones in 18 h cultures; Fig. 4) nitrite concentrations did not seem to affect bacterial motility (Table 2).

In viscous media, where lateral flagella were produced in addition to Fla, the motility of all the *A. brasilense* strains was the same in MPSS with NH₄Cl or with KNO₃, and it dropped to the decreased Sp245 level in Sp245.P3, but not in Sp245.P1 and Sp245.P5, in MPSS with KNO₂ (Table 2). Since not only flagellar rotation but also intercellular contacts [20] and other unknown factors are important for bacterial swarming, the diameter of the swarming rings formed by azospirilla in MPSS varied depending on the nitrogen source (Table 2). A decrease in the diameter of the swarming rings was observed in the series ammonium–nitrate–nitrite. Whereas in MPSS with NH₄Cl or KNO₃ the variants migrated faster than Sp245, in MPSS with KNO₂, their spreading rate was suppressed to the wild-type level. The only exception was Sp245.P5, which remained the best swimmer in all the three media (Table 2).

Thus, interstrain differences in growth and behavior of spontaneous variants of *A. brasilense* Sp245 with the same super-swarming phenotype in media supplemented with NH₄Cl ([19] and this study) could be revealed in the presence of other nitrogen sources—KNO₃ or KNO₂.

It is known that nitrate and nitrite reduction performed by *A. brasilense* Sp245 affects the plant–bacterial interactions and the growth of the host plants [12, 22]. As shown in this study, several spontaneous super-swarming derivatives of *A. brasilense* Sp245 have different DNA patterns

and behave differently in the presence of NO₃[−]/NO₂[−]. Such genomic and physiological plasticity of azospirilla is expected to be of significance for their survival in the rhizosphere and soil.

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