Chapter 7
Plasmid Plasticity in the Plant-Associated Bacteria of the Genus *Azospirillum*

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7.1 Introduction

The genus *Azospirillum* is a member of the class Alphaproteobacteria and the family Rhodospirillaceae. This genus was commonly described as gram-negative, nitrogen-fixing, oxidase-positive motile aerobic/microaerophilic bacteria with a DNA G + C content of about 70 mol% and with vibrioid, S-shaped, or helical cells which grow best on salts of organic acids (Tarrand et al. 1978). On the basis of phylogenetic, chemotaxonomic, physiological, and biochemical data, 15 *Azospirillum* species have been validly described to date (Table 7.1). Comparative analyses of the 16S rRNA gene sequences showed that *A. brasilense*, *A. canadense*, *A. rugosum*, and *A. palatum*; *A. lipoferum*, *A. halopraeferens*, *A. largimobile*, *A. doebereinerae*, *A. oryzae*, *A. melinis*, *A. zeae*, *A. picis*, and *A. thiophilum*; and *A. amazonense* and *A. irakense* form three groups of the most closely related *Azospirillum* species (Mehnaz et al. 2007a, b; Lin et al. 2009; Zhou et al. 2009; Lavrinenko et al. 2010).

Azospirilla occur worldwide in a broad range of complex and heterogeneous environments, including soil and the roots, stems, and leaves of cereals, forage grasses, vegetables, and many other plants. These bacteria have been widely used as models for studying the mechanisms of mutually beneficial associative plant–microbial interactions for the past 30 years.

All *Azospirillum* species are motile owing to the presence of flagella. Several species have a mixed type of flagellation. For example, in *A. brasilense*, *A. lipoferum*, and *A. irakense*, a single polar flagellum (Fla) is produced in liquid environments, and numerous lateral flagella (Laf) are induced in addition to Fla on viscous and solid media (Tarrand et al. 1978; Hall and Krieg 1983; Khammas et al. 1989). Fla is responsible for swimming, whereas Laf are used

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Table 7.1 List of validly described *Azospirillum* species

Azospirillum species	Type strain	Source	References
A. brasilense	Sp7	Rhizosphere of pangola grass (*Digitaria decumbens*), Brazil	Tarrand et al. (1978)
A. lipoferum	Sp59b	Roots of wheat (*Triticum aestivum*), Brazil	Tarrand et al. (1978)
A. amazonense	Y1	Roots of pangola grass (*Digitaria decumbens*), Brazil	Magalhães et al. (1983)
A. halopraeferens	Au4	Roots of Kallar grass (*Leptochloa fusca*), Pakistan	Reinhold et al. (1987)
A. irakense	KBC1	Roots of rice (*Oryza sativa*), Iraq	Khammas et al. (1989)
A. largimobile	ACM 2041	Water sample from a lake, Australia	Skerman et al. (1983), Ben Dekhil et al. (1997), Sly and Stackebrandt (1999)
A. doebereinerae	GSF71	Roots of Chinese silver grass (*Miscanthus sinensis*), Germany	Eckert et al. (2001)
A. oryzae	COC8	Rhizosphere of rice (*Oryza sativa*), Japan	Xie and Yokota (2005)
A. melinis	TMCY 0552	Stems and roots of molasses grass (*Melinis minutiflora*), China	Peng et al. (2006)
A. canadense	DS2	Rhizosphere of corn (*Zea mays*), Canada	Mehnaz et al. (2007a)
A. zeae	N7	Rhizosphere of corn (*Zea mays*), Canada	Mehnaz et al. (2007b)
A. rugosum	IMMIB AFH-6	Oil-contaminated soil, Taiwan	Young et al. (2008)
A. palatum	ww 10	Forest soil, China	Zhou et al. (2009)
A. picis	IMMIB TAR-3	Discarded road tar, Taiwan	Lin et al. (2009)
A. thiophilum	BV-S	Sulfur bacterial mat from a sulfide spring, Russia	Lavrinenko et al. (2010)

for swarming, i.e., the population's movement across wet surfaces (Hall and Krieg 1983). Chemotactic responses of motile *Azospirillum* strains toward seed and root exudates, a major source of nutrients and signals for rhizobacteria increase the probability that the bacteria find the plant in the soil. Binding of azospirilla to plant surfaces is a next essential phase in the interaction of these bacteria with their hosts. Polar flagella seem to have some adhesive properties and mediate relatively rapid and reversible adsorption of azospirilla to plant roots. Lipopolysaccharides (LPS), exopolysaccharides (EPS), and capsular polysaccharides (CPS) play a role in the subsequent firm anchoring of azospirilla to the plant root surface and in proliferation of these bacteria on the roots. The association of azospirilla with plants generally does not require invasion of plant tissues. However, some strains enter the plant and colonize the root and stem interior (recently reviewed by Barassi et al. 2007).

The well-known plant-growth-promoting activity of azospirilla (Okon and Labandera-Gonzalez 1994) is attributed to the synthesis of plant hormones

(auxins, cytokinins, and gibberellins), deamination of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC), production of nitrite and nitric oxide, nitrogen fixation, phosphate solubilization, biocontrol of phytopathogens, and other activities (for a recent review, see Bashan and de Bashan 2010). The use of a great number of *Azospirillum* strains (including *A. brasilense* Sp7, Cd, and Sp245; *A. lipoferum* Sp59b and 4B; and *A. irakense* KBC1) as plant inoculants under laboratory, greenhouse, and field conditions frequently resulted in acceleration of seed germination; enhancement of root, shoot, and leaf growth; increase in plant nitrogen and protein content and plant disease resistance; and several other beneficial effects (reviewed by Okon and Labandera-Gonzalez 1994; Lucy et al. 2004; Bashan and de Bashan 2010). However, the outcome of inoculation with the same *Azospirillum* strain varied significantly depending on the inoculant delivery system, soil type, climatic conditions, plant species, and many other parameters (Okon and Labandera-Gonzalez 1994; Lucy et al. 2004). Thus, a better understanding of the factors that influence the survival, ecological fitness, and plant-growth-promoting activities of azospirilla would be very useful.

It is accepted that the bacterial lifestyle influences genome size, content, and architecture (Bentley and Parkhill 2004). Accordingly, azospirilla have rather complex genomes. Although the *Azospirillum* genomes vary considerably in size, from 4.8 megabase pairs (Mb) in *A. irakense* to 9.7 Mb in *A. lipoferum* (Martin-Didonet et al. 2000), all of them consist of multiple replicons. Multipartite genomes are also typical of many other alphaproteobacteria, including those interacting with plants (Batut et al. 2004; MacLean et al. 2007). It was supposed that the presence of additional replicons may increase the adaptive potential of bacteria (González et al. 2006).

In *A. brasilense*, *A. lipoferum*, and *Azospirillum* sp. B510, a great number of genetic loci involved in the production of major cell-surface polymers (LPS, EPS, and CPS), components of the motility apparatus, and secondary metabolites important for the *Azospirillum*–plant interactions have been assigned to plasmids (Katzy et al. 1990, 1998; Vanbleu et al. 2004, 2005; Pothier et al. 2008; Prigent-Combaret et al. 2008; Lerner et al. 2009a, b; Kaneko et al. 2010; Katsy et al. 2010; Petrova et al. 2010b).

Several studies have recently shown that the genomes of *A. brasilense*, *A. lipoferum*, and *A. irakense* are quite dynamic and that some plasmids are involved in major genomic rearrangements (Katsy et al. 2002; Petrova et al. 2005a, b, 2010a; Vial et al. 2006; Pothier et al. 2008; Katsy and Prilipov 2009). In this review, I discuss what is currently known about plasmid plasticity in *Azospirillum* and how this genetic plasticity influences the bacterial phenotype.

7.2 General Features of Multipartite *Azospirillum* Genomes

A genomic resource for *Azospirillum* includes the complete genome sequence of the rice endophyte *Azospirillum* sp. B510 (Kaneko et al. 2010). Strain B510, isolated from the surface-sterilized stems of *Oryza sativa* cv. Nipponbare

(Elbeltagy et al. 2001) is closely related to *A. oryzae* COC8 (Xie and Yokota 2005; Kaneko et al. 2010). Inoculation of rice seedlings with *Azospirillum* sp. B510 significantly increased the growth of newly generated leaves and shoot biomass in a greenhouse, as well as tiller numbers and seed yield under paddy field conditions (Isawa et al. 2010). Inoculated rice plants became more resistant to diseases caused by the phytopathogenic fungus *Magnaporthe grisea* and the bacterium *Xanthomonas oryzae* via an unclear mechanism (Yasuda et al. 2009). It should be noted, however, that in field experiments, the effects of *Azospirillum* sp. strain B510 inoculation on the growth of different rice cultivars varied depending on the rice genotype and nitrogen level (Sasaki et al. 2010).

The results of two other projects, focused on the genomes of *A. brasilense* Sp245 (http://genome.ornl.gov/microbial/abra/), a strain isolated from surface-sterilized wheat roots (Baldani et al. 1983), and *A. lipoferum* 4B (http://www.genoscope.cns.fr/), isolated from the rhizosphere of rice (Thomas-Bauzon et al. 1982), have not been published yet.

7.2.1 Plasmids

Plasmids are extrachromosomal replicons that often facilitate adaptations of their hosts to new and hostile environments (Kado 1998). Practically all analyzed *Azospirillum* strains possessed plasmids, ranging in size from 6 kilobase pairs (kb) to more than 1.8 Mb (Martin-Didonet et al. 2000; Kaneko et al. 2010). The plasmid complement seems to play an important role in the beneficial *Azospirillum*–plant associations (Table 7.2).

The genome of *Azospirillum* sp. B510 is arranged into seven replicons: a chromosome of 3.31 Mb and six plasmids, pAB510a–pAB510f, of 0.26–1.46 Mb (Kaneko et al. 2010). Notably, the plasmid constituent of the *Azospirillum* sp. B510 genome encodes more proteins than the chromosome (Table 7.3).

The presence of ribosomal RNA genes on several high-molecular-weight replicons (Caballero-Mellado et al. 1999; Martin-Didonet et al. 2000; Kaneko et al. 2010) supports the idea that at least some *Azospirillum* plasmids should be regarded as "minichromosomes" (Wood et al. 1982). Even a not very large, ~90-MDa (~159-kb) plasmid (p90, or pRhico) (Vanbleu et al. 2004) could not be eliminated from *A. brasilense* Sp7 cells, suggesting that it is essential for the viability of the host strain (Onyeocha et al. 1990).

Plasmids of *A. brasilense*, *A. lipoferum*, and *A. irakense* are prone to frequent rearrangements, leading to the appearance of bacteria with individual plasmid profiles (Matveev et al. 1987; Katsy et al. 1990, 2002; Petrova et al. 2005a, 2010a; Vial et al. 2006). Differences in plasmid contents of the same *Azospirillum* strain kept in different laboratories (Caballero-Mellado et al. 1999; Martin-Didonet et al. 2000; Pothier et al. 2008) could also be attributed (at least in part) to plasmid plasticity. For example, an 85-MDa plasmid (p85) of *A. brasilense* Sp245 and

Table 7.2 Known plasmid-encoded traits potentially important for beneficial *Azospirillum*–plant interactions

Bacterial phenotype	*Azospirillum* strain	Plasmid involved	References	Possible role in *Azospirillum*–plant interaction
Flagellation, motility, and chemotaxis	*A. brasilense* Sp7	pRhico (p90)	Vanbleu et al. (2004)	Bacterial movement toward the plant
	A. brasilense Sp245	p85 and p120	Katzy et al. (1998, 2001), Borisov et al. (2009)	
	Azospirillum sp. B510	pAB510a, pAB510b, pAB510c, pAB510d, and pAB510e	Kaneko et al. (2010)	
	A. brasilense Sp7	pRhico	Vanbleu et al. (2004, 2005), Lerner et al. (2009a, b)	
	A. brasilense Sp245	p85 and p120	Katzy et al. (1998), Borisov et al. (2009), Katsy et al. (2010)	
Cell-envelope and motility apparatus components	*Azospirillum* sp. B510	pAB510a, pAB510d, pAB510e, and pAB510f	Kaneko et al. (2010)	Bacterial establishment on plant surfaces
	A. brasilense Sp245	p85	Katzy et al. (1990)	
Indole-3-acetic acid production	*Azospirillum* sp. B510	pAB510b	Kaneko et al. (2010)	Auxin stimulation of plant growth
	Azospirillum sp. B510	pAB510b	Kaneko et al. (2010)	
ACC-deaminase activity	*A. lipoferum* 4B	a 750-kb plasmid	Prigent-Combaret et al. (2008)	Lowering of plant ethylene levels
	A. brasilense Sp7	p115	Petrova et al. (2010b)	
	A. brasilense Sp245	p85	Petrova et al. (2010b)	
		250-kb (a probable cointegrate of p85 with another plasmid) and ~750-kb		
Nitrite reduction and NO formation	*A. brasilense* Sp245	plasmids	Pothier et al. (2008)	Plant root growth promotion by NO
	Azospirillum sp. B510	pAB510c	Kaneko et al. (2010)	
Uptake of siderophores	*Azospirillum* sp. B510	pAB510a, pAB510d, and pAB510e	Kaneko et al. (2010)	Reduction of iron available to phytopathogens

a 115-MDa plasmid (p115) of *A. brasilense* Sp7 are frequently absent from the plasmid profiles of Sp245 and Sp7 clones. These low-copy-number plasmids (Katsy 1992; Holguin et al. 1999) are incompatible (Katsy 1992) suggesting a common plasmid maintenance mechanism. Preliminary data presume that p85 and p115 could reversibly integrate into other *A. brasilense* replicons (Petrova et al. 2005a, b; Katsy and Prilipov 2009).

Table 7.3 Some features of the sequenced *Azospirillum* sp. B510 genome[a]

GenBank accession number	Circular DNA molecule	Length (kb)	GC content (%)	Coding DNA (%)	Predicted protein encoding genes	IS elements[b]	Prophages	Genomic islands
AP010946	Chromosome	3,311.4	67.8	86	2,893	77	2	6
AP010947	pAB510a	1,455.1	67.6	88	1,131	57	–	–
AP010948	pAB510b	723.8	67.5	89	631	78	–	–
AP010949	pAB510c	681.7	67.4	89	533	20	–	–
AP010950	pAB510d	628.8	68.0	87	519	7	–	1
AP010951	pAB510e	537.3	67.5	87	415	11	–	1
AP010952	pAB510f	261.6	65.9	90	187	30	–	–

[a]Compiled from GenBank records AP010946–AP010952 and from Kaneko et al. (2010)
[b]Total number of complete and partial IS elements

7.2.2 Insertion Sequences

As mentioned above, *Azospirillum* genomes seem to be highly dynamic entities. A significant source of genomic plasticity could be the transposition of insertion sequence (IS) elements, which are widespread in bacteria (Mahillon and Chandler 1998). Transpositions of IS elements may cause gene inactivation or activation of silent genes. Recombination between homologous copies of IS elements provokes DNA inversions or deletions (Mahillon and Chandler 1998). Besides, IS elements can mediate horizontal gene transfer (Heuer and Smalla 2007).

Two IS elements of the *A. brasilense* Sp245 plasmid p85, IS*Azba1* (distantly related to the IS*256* family) and IS*Azba3* (from the IS*5* family/IS*903* group), mediate fusions of p85 with foreign plasmids (Katsy and Prilipov 2009). These IS elements seem to be powerful natural tools useful for the enrichment of the *A. brasilense* genome with novel genetic material. As other factors potentially contributing to the known genetic plasticity of p85, truncated IS*Azba2* of the IS*L3* family and a phage integrase gene were also identified in this plasmid (Katsy and Prilipov 2009).

Two IS elements found on pRhico of *A. brasilense* Sp7 are homologous to the *Bradyrhizobium japonicum* IS elements ID145 and ID270, encoding a transposase and a DNA invertase (Vanbleu et al. 2004).

A striking feature of the *Azospirillum* sp. B510 genome is the abundance of multiple copies of IS elements (Table 7.3). The B510 genome contains 139 complete and 141 truncated copies of IS elements from at least 12 families (IS*3*, IS*5*, IS*21*, IS*66*, IS*110*, IS*256*, IS*630*, IS*701*, IS*1380*, IS*As1*, IS*L3*, and ISNCY). Many of these IS elements are present in more than one copy on the same or on different replicons (Kaneko et al. 2010) and thus represent potential sites for DNA recombination. The presence of redundant IS elements suggests their important role in the *Azospirillum* genome rearrangements.

7.2.3 Prophages

Many bacteriophages contribute to bacterial diversity owing to transduction of new genes and lysogenic conversion (Chibani-Chennoufi et al. 2004).

Several temperate strain-specific *Azospirillum* bacteriophages are known (Elmerich et al. 1982; Germida 1984; Boyer et al. 2008). Interestingly, in stable lysogens of *A. lipoferum* SpBr17, prophage Al-1 was supported as a 22-MDa (~37-kb) plasmid (Elmerich et al. 1982).

Phages isolated from cell lysates after mitomycin C treatment of *A. brasilense* cultures have 62–65-kb genomes (Boyer et al. 2008). In the closely related *A. brasilense* strains Cd and Sp7, practically identical 62.3-kb prophages reside in ~376-MDa (570-kb) plasmids; in addition, the 27.3-kb fragments of these prophages are duplicated in the 90-MDa plasmids (pRhico) (Boyer et al. 2008). In an almost complete nucleotide sequence of pRhico from *A. brasilense* Sp7, six putative prophage genes were identified (Vanbleu et al. 2004).

In *Azospirillum* sp. B510, two diverse prophages span 66.7 and 31.8 kb of the chromosome; an altered copy of the 66.7-kb prophage also resides in the chromosomal DNA (Kaneko et al. 2010).

Since the phages induced from *A. lipoferum*, *A. doebereinerae*, and *Azospirillum* sp. B510 (Boyer et al. 2008; Kaneko et al. 2010) had very small (ca. 10-kb) genomes, they were suggested to be the phage-like gene-transfer elements (GTE) (Boyer et al. 2008). However, no transducing activity has been recorded in any *Azospirillum* phage or GTE yet.

7.2.4 Genomic Islands

The contents of bacterial genomes can be changed quickly and significantly because of horizontal gene transfer and subsequent integration of foreign genetic elements into resident DNAs, where they form "genomic islands" (Dobrindt et al. 2004).

Apart from selfish mobility loci, genomic islands harbor other genes with specific functions. Expression of those genes can change bacterial behavior, increase bacterial fitness, competence in interacting with higher organisms, and adaptability to new environments ("fitness," "ecological," "saprophytic," "symbiosis," "pathogenicity," and other islands) (Dobrindt et al. 2004; Heuer and Smalla 2007).

Sequence analysis of the *Azospirillum* sp. B510 genome revealed six chromosomal and two plasmid (located in pAB510d and pAB510e) regions with typical signs of genomic islands (Kaneko et al. 2010). All the eight regions were located between duplicated portions of tRNA genes; possessed putative genes for integrases, site-specific recombinases, and transposases; and differed from the core genome in G + C content. The sizes of those genomic regions varied from 6.7 kb (chromosomal B510GI03) to 71 kb (B510GIe8 in pAB510e) (Kaneko et al. 2010).

Besides several loci coding for an integrase, transposases, and hypothetical proteins, a 22.6-kb genomic island (B510GId7) from pAB510d includes predicted genes for a transcriptional regulator and two-component sensor histidine kinase and response regulator (GenBank accession number AP010950).

In addition to numerous mobility and recombination loci, the 71-kb genomic island B510GIe8 possesses genes encoding a transcriptional regulator, two-component sensor histidine kinases and response regulators, a hemolysin-type calcium-binding protein, H^+-transporting and Mg^{2+}-importing ATPases, the chemotaxis protein methyltransferase, glycosyltransferases, and other predicted proteins (GenBank accession number AP010951). The genomic islands probably provide *Azospirillum* sp. B510 with some selective advantages.

7.3 Plasmid Dynamics and Phenotypic Variations in Several *Azospirillum* Species

Soil bacteria are supposed to use genetic and phenotypic diversification for survival in harsh and fluctuating environments. Various genomic rearrangements, often involving mobile genetic elements, may be induced in bacteria under specific growth conditions, such as prolonged starvation and oxidative and temperature stresses (Rajeshwari and Sonti 2000; Faure et al. 2004; Foster 2007). The presence of numerous plasmids, IS elements, phage-associated genes, and genomic islands in the *Azospirillum* genomes strongly suggests that these mobile arsenals could often be responsible for the phenotypic and genomic plasticity observed in these bacteria (see below).

7.3.1 Azospirillum brasilense

Azospirilla produce various cell-surface glycopolymers: LPS, EPS, and CPS. In most gram-negative bacteria, LPS consists of the hydrophobic lipid A, a core oligosaccharide, and an O-specific polysaccharide (OPS). A "smooth-type" LPS (S-LPS) possesses all the three structural entities; a "rough-type" LPS (R-LPS) is devoid of OPS; and SR-LPS is formed by lipid A-core capped with a single OPS unit (Raetz and Whitfield 2002).

Multiple open reading frames apparently responsible for the synthesis of LPS, EPS, CPS, and other cell-surface biopolymers were revealed in pRhico of *A. brasilense* strain Sp7 (Vanbleu et al. 2004, 2005; Lerner et al. 2009a, b). These findings may explain why it was not possible to cure the Sp7 cells from pRhico (Onyeocha et al. 1990).

Almost nothing is known about the functions of other *A. brasilense* Sp7 plasmids. However, plasmid rearrangements were found that were accompanied

by alterations in a number of Sp7 traits (Matveev et al. 1987; Petrova et al. 2005a, b, 2010a).

For example, indirect data on the possible involvement of the p115 plasmid in the regulation of bacterial R–S dissociation were obtained (Matveev et al. 1987). After prolonged incubation of *A. brasilense* Sp7 at 40–42°C, a fairly stable variant, Sp7-S, was identified, that had lost the 115-MDa replicon. The replicon loss correlated with persistence of the S phenotype in Sp7-S, i.e., the formation of smooth colonies (Matveev et al. 1987).

The differences between the R- and S-variants of Sp7 presumably resulted from the age-dependent changes in the contributions of two full-length LPS to the architecture of the colony surface rather than from partial or complete loss of OPS (Matora et al. 2003).

After several years of storage of *A. brasilense* Sp7-S at −70°C, its R-, SR-, and S-variants Sp7.1–Sp7.9 were isolated that contained derivatives of pRhico with changed molecular weights (94, 107, 121, 124, and 131 MDa) and structures (Petrova et al. 2005a, 2010a). For example, in *A. brasilense* Sp7.2 and Sp7.4, the new 131-MDa plasmid showed homology to both pRhico and p115 and seemed to be a hybrid of pRhico and a segment of p115 (Petrova et al. 2005a).

It was suggested that at least part of p115 has integrated into the chromosome of the variant Sp7-S, making the genome unstable and prone to DNA rearrangements (Petrova et al. 2005a). The genetic instability of *A. brasilense* Sp7-S may also be due to the specific method of its derivation (incubation at elevated temperature) (Matveev et al. 1987). It should be noted that soil bacteria are often exposed to elevated temperatures in their natural habitats. Upon multiple freezing–thawing of the *A. brasilense* Sp7 culture (which is also quite probable in soil ecosystems), an S-variant, Sp7.K2, was isolated that had also lost p115 (Petrova et al. 2005a).

A close relative of Sp7, *A. brasilense* strain Cd, lacking p115 (Petrova et al. 2005b) was isolated from the roots of bermuda grass after inoculation with Sp7 (Eskew et al. 1977). Both strains were characterized by nearly identical two-dimensional protein maps and DNA restriction fragment length polymorphisms (RFLP) (De Mot and Vanderleyden 1989; Gündisch et al. 1993; Petrova et al. 2005b). However, subtle differences were revealed in the LPS antigenic structures of Sp7 and Cd (Kirchhof et al 1997; Petrova et al. 2005b). Unlike in *A. brasilense* Sp7-S, further plasmid rearrangements were not detected in *A. brasilense* Cd. In contrast to *A. brasilense* Sp7, Sp7-S, and other *Azospirillum* strains, colonies of strain Cd do not dissociate on minimal synthetic media and retain their S-phenotype over a long time (Petrova et al. 2005b). The genetic causes for the differences in colony morphology and LPS structure in the closely related strains Sp7 and Cd, characterized by distinct plant-associated backgrounds, are of interest.

All the studied phenotypic variants of *A. brasilense* Sp7 retained the ability to swim and swarm. Moreover, the rates of their spreading in soft media were 1.7–2.7 times higher than that in the parent strain Sp7. Several variants of Sp7 have increased resistance to ampicillin and to cationic (cetyltrimethylammonium bromide) and anionic (sodium dodecyl sulfate) surface-active agents. The capability of a

number of Sp7 variants to grow on a minimal medium with cetyltrimethylammonium bromide as the sole carbon source deserves further investigation (Petrova et al. 2005a, b).

Lerner et al. (2010) isolated spontaneous derivatives of *A. brasilense* $Sp7^{-}$, a non-aggregating variant of Sp7, after 12-day starvation ($Sp7_{E}$ and two other variants) or colonization of maize roots (one variant). These derivatives differed from the parent strain in several traits. According to preliminary data, a plasmid rearrangement occurred in the variant $Sp7_{E}$. As compared to *A. brasilense* $Sp7^{-}$ and Sp7, the EPS-overproducing variant $Sp7_{E}$ has modified LPS and EPS, increased resistance to environmental stresses and to some antibiotics, and enhanced biofilm-forming capability (Lerner et al. 2010).

In natural environments, bacteria mainly exist in biofilms, i.e., structured communities embedded into a polymeric extracellular matrix and located at the interface between two media (Nikolaev and Plakunov 2007). The efficiency of biofilm formation by *A. brasilense* Sp7 and its variants was analyzed by Petrova et al. (2010a). It turned out that in Sp7, the above-described plasmid rearrangements (Petrova et al. 2005a) negatively affected biofilm formation on hydrophobic (polystyrene) and (less frequently) hydrophilic (glass) abiotic surfaces (Petrova et al. 2010a).

The *A. brasilense* Sp7 variants lacking p115 and containing the modified pRhico were also less active in plant root colonization during the first hours of interaction (Petrova et al. 2010a). Four to five hours after inoculation of wheat seedlings, the cells of Sp7 actively colonized root tips, hairs, and root fractures. On the next day, the Sp7 biofilms became more compact and multilayered, with cell aggregates starting to form. The overall picture of wheat root colonization by the variants of Sp7 was similar to that observed in the parent strain; however, at the initial steps of formation, their biofilms were thinner (especially in the cases of Sp7.K2 and Cd), single cells could be detected, and no colonization was observed in surface regions remote from the root tip. After 24 h, the wild-type Sp7 strain and all its variants formed cell aggregates on wheat roots; most of them were located on root hairs and tips and in places of root hair initiation, and visible interstrain differences disappeared (Petrova et al. 2010a). Thus, it seems that coordinated expression of the complete set of plasmid genes is important for more rapid adaptation of *A. brasilense* Sp7 to the new environments and for more successful realization of the biofilm development program.

As distinct from many other *Azospirillum* strains colonizing only plant surfaces, *A. brasilense* Sp245 is capable of invading plant roots (Baldani et al. 1983; Assmus et al. 1995). This strain has high potential for producing phytohormones and inducing the branching of roots and root hairs (Jain and Patriquin 1984).

A. brasilense Sp245 possesses LpsI and LpsII with subtle differences between their OPSs and/or core oligosaccharides recognizable with polyclonal antibodies raised against the wild-type LPS (Katzy et al. 1998) and by ion-exchange chromatography (Fedonenko et al. 2004). The OPSs of both LPSI and LPSII are composed of identical pentasaccharide D-rhamnose (D-Rha) repeating units (Fig. 7.1) (Fedonenko et al. 2002).

1
→2)-β-*D*-Rha*p*-(1→3)-α-*D*-Rha*p*-(1→3)-α-*D*-Rha*p*-(1→2)-α-*D*-Rha*p*-(1→2)-α-*D*-Rha*p*-(1→

2
→6)-α-*D*-Gal*p*NAc-(1→4)-β-*D*-Man*p*NAcA-(1→

Fig. 7.1 Structures of the repeating units of the O-specific polysaccharides of *A. brasilense* Sp245 (1) and its variant Sp245.5 (2), which spontaneously lost plasmids p85 and p120 upon the formation of a new, ~300-MDa megaplasmid (Fedonenko et al. 2002, 2010)

A 120-MDa plasmid (p120) of *A. brasilense* Sp245 encodes a number of predicted glycosyltransferases participating in the biosynthesis of LPSI, LPSII, and Calcofluor-binding polysaccharides (CBPS, Cal^+ phenotype), which include both EPS and CPS fractions (Katzy et al. 1998; Katsy et al. 2010).

Interestingly, the same penta-D-rhamnan OPSs were identified in *A. brasilense* SR75 (Fedonenko et al. 2005), Sp107, and S27 and in *A. lipoferum* RG20a (Boiko et al. 2010). At least in two of these strains (*A. brasilense* SR75 and Sp107), 120-MDa plasmids contain a region that is highly homologous to a 14-kb segment of p120 coding for the LPS biosynthesis enzymes. On the other hand, in the genomes of *A. brasilense* SR75 and Sp107, no homology to another (~8.3-kb) segment of p120 was found (Fedonenko et al. 2005; Katsy et al. 2010).

The p85 plasmid of *A. brasilense* Sp245 is known to be involved in tryptophan metabolism, related to auxin production (Katzy et al. 1990), and to encode predicted glycosyltransferases (Katsy et al. 2010), denitrification enzymes (Petrova et al. 2010b), and some other proteins (Katsy and Prilipov 2009; Petrova et al. 2010b).

After an analysis of the plasmid profiles of *A. brasilense* Sp245 strains obtained from different laboratories, Pothier et al. (2008) concluded that the smallest replicon (i.e., p85) probably underwent various independent rearrangements: a deletion of ~10 MDa, a cointegration with another plasmid, and a complete loss by the strain used in the Sp245 genome-sequencing project.

A variant of *A. brasilense* Sp245, named Sp245.5, was characterized that had spontaneously lost p85 and p120 upon the formation of a new ~300-MDa megaplasmid after long-term storage of the bacteria in a rich medium (Katsy et al. 1994, 2002). The wild-type (Sp245) and derivative (Sp245.5) strains have the same acetylene reduction activity; nearly identical SDS-PAGE profiles of the outer membrane proteins, except for an additional 44-kDa protein in Sp245.5 (Katsy et al. 1994); and similar RFLP patterns, except for the loci probably involved in plasmid rearrangement (Katsy et al. 2002). As distinct from the parent *A. brasilense* strain Sp245, Sp245.5 is unable to reduce nitrite and produces about two times less indole-3-acetic acid in the presence of tryptophan (Katzy et al. 1995).

Furthermore, strain Sp245.5 has drastic alterations in the biosynthesis of CBPS (Cal^- phenotype) and possesses a highly heterogeneous LPS, which could not be recognized by polyclonal antibodies raised against the LPS of Sp245 (Katsy et al. 2002). The macromolecular organization of the Sp245.5 LPS differs distinctly from that of the Sp245 LPS. Whereas the core oligosaccharide of the wild-type strain shows a very high degree of substitution with polysaccharide chains, attesting to the

prevalence of S-LPS in the LPS pool (Fedonenko et al. 2002), Sp245.5 displays both S- and R-LPS at an approximate ratio of 2:1 (Fedonenko et al. 2010).

The repeating unit of the OPS of *A. brasilense* Sp245.5 is a disaccharide consisting of residues of *N*-acetyl-D-galactosamine and *N*-acetyl-D-mannosaminuronic acid (Fig. 7.1), which has not been hitherto found in *Azospirillum* (Fedonenko et al. 2010).

Thus, the OPS of *A. brasilense* Sp245.5 differs from the D-rhamnan OPS of the parent strain Sp245 (Fedonenko et al. 2002) in both composition and structure (Fedonenko et al. 2010). It should be noted that *N*-acetyl-D-mannosaminuronic acid is a rare sugar, which, for instance, is present in the enterobacterial common antigen, a cyclic or linear heteropolysaccharide (Lugowski et al. 1983).

The loss of the D-rhamnan OPS by *A. brasilense* Sp245.5 could result from the adaptation of this bacterium to the conditions of several years' storage in a rich medium at room temperature. Probable activation of mobile elements and genetic rearrangements under the above storage conditions could lead to the deletion and/or inactivation of the D-rhamnan LPS biosynthesis genes with simultaneous activation of silent genes and a pathway for the production of OPS with novel monosaccharide composition and structure of the repeating unit.

The dramatic change in the LPS structure correlates with the activation of biofilm formation by *A. brasilense* Sp245.5 on hydrophilic and hydrophobic surfaces. Biofilms formed by its cultures are significantly more pronounced than those of the parent strain Sp245 (Fig. 7.2) (Sheludko et al. 2008).

A. brasilense Sp245.5 retained the flagellation and swimming motility pattern of the wild-type strain. However, its cells swim slower (at a speed of 17.1 ± 0.8 μm/s) than the cells of Sp245 (29.3 ± 0.9 μm/s). On semisolid media, populations of the Sp245.5 cells form "diffuse" spreading zones, whereas the wild-type bacteria form distinct swarming rings. Most probably, the altered spreading phenotype of Sp245.5 is caused by the principal change in the structure of cell-surface polysaccharides, which unbalances important carbohydrate–carbohydrate and protein–carbohydrate interactions (Shelud'ko et al. 2009).

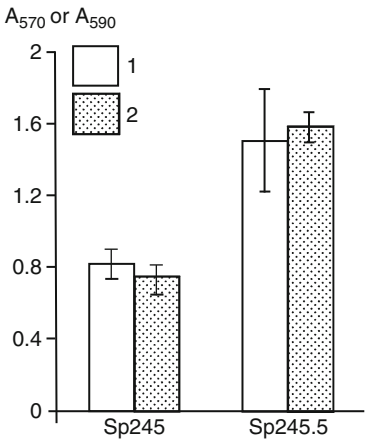

Fig. 7.2 Relative amount of biomass in the *A. brasilense* Sp245 and Sp245.5 biofilms formed on the surfaces of polystyrene (A_{570}) (1) and glass (A_{590}) (2) upon 96-h incubation at 28°C. Crystal violet staining of the biofilms was used. Details of the experiments can be found in Sheludko et al. (2008)

Thus, the spontaneous change in the content and structure of the *A. brasilense* Sp245 plasmids profoundly affects the metabolism, cell-surface polymers, and social activities of this bacterium.

A. brasilense WN1, a strain associated with wheat (Blaha et al. 2006), harbors plasmids of approximately 190, 260, 500, 570, and 650 kb (Vial et al. 2006). This strain showed the capacity for production of stable immotile variants (at a frequency of 3.6×10^{-3} per cell per generation), a process concurrent with a loss of the 260-kb replicon (Vial et al. 2006).

7.3.2 *Azospirillum lipoferum*

Under laboratory conditions, the strain 4B of *A. lipoferum* frequently produces a stable phase variant, named $4V_I$ (Alexandre and Bally 1999). In distinction to the wild-type strain, the $4V_I$ variant assimilates other carbohydrates; lacks a polar flagellum and does not swim; constitutively produces lateral flagella and has enhanced swarming capacity; and does not reduce triphenyltetrazolium chloride (Alexandre and Bally 1999; Alexandre et al. 1999) or nitrous oxide (Vial et al. 2006). When grown at extremely low oxygen concentrations, *A. lipoferum* $4V_I$ produces a stable laccase-positive melanin-producing atypical variant named $4V_{II}$. Neither *A. lipoferum* $4V_I$ nor $4V_{II}$ is able to revert to the wild-type phenotype (Alexandre and Bally 1999).

A. lipoferum 4B was isolated from the rice rhizosphere simultaneously with the immotile laccase-positive strain *A. lipoferum* 4T (Bally et al. 1983), which turned out to be very similar to the variant $4V_{II}$. Proven to be closely related to *A. lipoferum* 4B (Haurat et al. 1994; Vial et al. 2006), *A. lipoferum* 4T was supposed to be a spontaneous variant of 4B (the motile, laccase-negative strain) generated via a two-step phenotypic switching event within the rice rhizosphere (Alexandre and Bally 1999). *A. lipoferum* 4T retained the ability of 4B to efficiently colonize rice roots, but unlike the motile 4B, it needs the roots to be stabilized in sterile soil (Alexandre et al. 1996). Both *A. lipoferum* 4B and 4T coexist in sterile soil and the rice rhizosphere. In the rhizosphere of rice, the frequency of the appearance of stable immotile variants of *A. lipoferum* 4B (similar in other traits to the parent strain) seemed elevated (Alexandre et al. 1996).

In *A. lipoferum* 4B, replicons of 40, 310, 460, 700, 750, ~1,000, ~1,600, and ~2,200 kb were identified (Vial et al. 2006). Both *A. lipoferum* 4T and V_I retained all the replicons of 4B, except for the 750-kb plasmid. Interestingly, this plasmid harbors the *acdS* gene, a predicted product of which deaminates the ethylene precursor ACC and may be involved in determining the plant-growth-promoting activity of *A. lipoferum* 4B (Prigent-Combaret et al. 2008).

A new plasmid of 400 kb, nonhomologous to the lost 750-kb replicon, was detected in *A. lipoferum* 4T (Vial et al. 2006). It remains to be established whether the 400-kb plasmid was present in an integrated form in the genome of *A. lipoferum* 4B or was acquired by strain 4T via horizontal gene transfer from other rhizosphere bacteria.

7.3.3 *Azospirillum irakense*

A. irakense KBC1, a strain isolated from the rhizosphere of rice (Khammas et al. 1989), possesses megaplasmids of approximately 1,050 and 1,400 kb (Vial et al. 2006). At a frequency of up to 10^{-4} per cell per generation, KBC1 produces unstable immotile variants, the subculturing of which gives a mixture of wild-type and variant colonies (at a ratio of about 10 to 1, respectively). The phenotypic switches correlate with a supposed reversible split of the 1,400-kb replicon into 160-kb and 1,240-kb plasmids (Vial et al. 2006).

7.4 Concluding Remarks

As discussed above, in several strains of *A. brasilense*, *A. lipoferum*, and *A. irakense*, spontaneous DNA rearrangements were accompanied by changes in the structure of the major cell-surface polymers, bacterial motility, biofilm-forming capability, resistance to xenobiotics, and other traits potentially important for bacterial survival in soil and for colonization of plants. It is highly probable that the natural intrastrain diversification of azospirilla is the key to their success as ubiquitous environmental bacteria. However, the ecological consequences of the *Azospirillum* plasmid (and, on the whole, genome) plasticity have not been comprehensively studied yet. It seems important to find out how the profound genomic and phenotypic plasticities influence the adaptation of azospirilla to the complex environments such as soil and the phytosphere, as well as their plant-growth-promoting ability.

A challenge lies in determining the molecular mechanisms responsible for frequent genomic rearrangements. The accumulation of several *Azospirillum* genome sequences and the development of new experimental tools for the examination of intercellular and single-DNA-molecules variability (Medini et al. 2008; McCaughan and Dear 2010) are expected to yield more comprehensive knowledge about bacterial genomes as fluid and dynamic entities. These insights would have broader implications for understanding the natural behavior of other rhizobacteria, also prone to phenotypic switches (Achouak et al. 2004; Espinosa-Urgel 2004; Martínez-Granero et al. 2005) and for competent managing of sustainable plant–bacterial associations.

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