**Genome Structure of the Genus *Azospirillum***

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**Azospirillum** species are plant-associated diazotrophs of the alpha subclass of Proteobacteria. The genomes of five of the six *Azospirillum* species were analyzed by pulsed-field gel electrophoresis. All strains possessed several megareplicons, some probably linear, and 16S ribosomal DNA hybridization indicated multiple chromosomes in genomes ranging in size from 4.8 to 9.7 Mbp. The *nifHDK* operon was identified in the largest replicon.

Despite several reports describing the presence of plasmids in *A. brasilense* and *A. lipoferum* (7, 19, 21, 22, 31), information about their genome size is imprecise to date. In 1982, Wood et al. (31), using a modified Eckhardt electrophoresis method, described the presence of several very large DNA bands with molecular sizes up to 2.8 Mbp that they called minichromosomes, based on their apparent large molecular size. They estimated the *Azospirillum* genome size as 1.8 times larger than that of *Escherichia coli*.

In this paper, we describe the presence of several megareplicons in 10 strains of five *Azospirillum* species with molecular sizes ranging from 0.2 to 2.7 Mbp as determined by pulsed-field gel electrophoresis (PFGE). The PFGE DNA patterns differ within the same species, which indicates that they are strain specific. In all strains tested, the presence of 16S rDNA was detected in more than one replicon, suggesting that *Azospirillum* contains multiple chromosomes. Also, the PFGE behavior indicates that some of the replicons are probably linear DNA molecules.

The *Azospirillum* species analyzed were *A. brasilense*, strains Sp7 (ATCC 29145) (30), Cd (ATCC 29710) (29), FP2 (20), and Sp245 (2); *A. lipoferum*, strains Sp59b (ATCC 29707) (30) and JA25 (5); *A. amazonense*, strains Y2 (ATCC 35120) and Y6 (ATCC 35121) (16); *A. irakense* (11); and *A. halopraeferens* (23). All bacterial strains were grown in NFbHP-malate (12) or DYGS medium (2 g of glucose/liter, 2 g of malic acid/liter, 1.5 g of peptone/liter, 2 g of yeast extract/liter, 0.5 g of K$_2$HPO$_4$/liter, 0.5 g of MgSO$_4$ · 7H$_2$O/liter, 1.5 g of glutamic acid/liter [pH 6.8]) at 30°C in a rotary shaker, except for *A. irakense* and *A. halopraeferens*, which were grown at 37°C.

The intact genome of *Azospirillum* was analyzed by PFGE (27). The cells were grown in liquid medium to an optical density at 600 nm ranging from 0.2 to 0.7, depending on the strain, and chromosomal DNA was purified as described previously (14) and analyzed in agarose gels (1.2%) using a Gene Navigator pulsed-field system (Pharmacia).

Bacterial cells were embedded in 100 µl of low-melting-point agarose in buffer SET (50 mM Tris-HCl [pH 7.5], 20 mM EDTA, 200 mM NaCl). Lysis was achieved by using a lysis solution (10 mM Tris [pH 7.5], 50 mM NaCl, 100 mM EDTA, 0.2% deoxycholate, 0.5% N-lauryl sarcosine) with lysozyme (1 mg/ml) at 37°C for 24 h. Protein digestion was carried out using proteinase K (0.1 mg/ml) in 0.5 mM EDTA (pH 8.0) and 1% N-lauryl sarcosine at 52°C for 48 h.

The *A. brasilense* strains analyzed showed five megareplicons, ranging in size from 0.63 to 2.5 Mbp (Fig. 1 and Table 1). The strains FP2, Sp7, and Cd showed the same DNA profile...
showed 8 and 10 replicons, respectively, with molecular sizes ranging from 0.15 to 2.6 Mbp, and none of the replicons seemed to comigrate (Fig. 1 and 2 and Table 1). Both *A. amazone*n*ens* strains, Y2 and Y6, showed four replicons but had distinct PFGE profiles, with sizes varying from 0.71 Mbp (Y6) to 2.8 Mbp (Y2). Several replicons were also observed in *A. halopraeferens* and *A. irakense* (Fig. 2 and Table 1), each strain with a specific DNA pattern. This is the first report of the presence of megareplicons in *A. amazone*n*ens*, *A. irakense*, and *A. halopraeferens*.

The overall genome size of members of the genus *Azospirill*um varied from a minimum of 4.8 Mbp (*A. irakense*) to 9.7 Mbp (*A. lipoferum* strain Sp59b). These results indicate that the organization of the *Azospirill*um genome is highly complex, with the genetic information distributed on several replicons. In addition, the DNA pattern was strain specific rather than species specific, a result also observed for *Brucella* (9). The role of these replicons in the ecological and in vitro survival of these species remains to be determined but may support the exceptional ecological distribution and metabolic flexibility of members of this genus (9).

According to Römling et al. (24), only linear DNA molecules permeate a gel and can be separated by PFGE. Large circular DNA molecules do not permeate the gel and can be analyzed only after linearization either by physical or enzymatic treatment or randomly during DNA preparation. Because the bacterial lysis conditions are made very mild to avoid DNA breakage, randomly broken DNA produced from circular molecules is in low concentration and therefore shows weak, less intense bands, which also vary with the method of preparation, on a PFGE gel. The two largest replicons of *A. brasilense* and *A. lipoferum* showed less intensity than the others bands (Fig. 1 and 2), and their relative intensities varied with the method of preparation (data not shown), suggesting that those replicons were circular DNA molecules. In addition, partial DNA digestions of strains FP2 and JA25 with very low concentrations of restriction enzymes to produce a single cut per molecule caused an increase in the intensities of those bands relative to the others, confirming that they were produced from circular DNA (data not shown). A different be-

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**TABLE 1.** Molecular size of replicons of *Azospirillum* spp., as determined by PFGE

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Sizes (Mbp) of Replicons</th>
<th>Genome (estimated)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. brasilense</em></td>
<td>FP2</td>
<td>2.5&lt;sup&gt;a&lt;/sup&gt;; 1.72&lt;sup&gt;a&lt;/sup&gt;; 0.81&lt;sup&gt;a&lt;/sup&gt; (L); 0.7 (L); 0.63&lt;sup&gt;a&lt;/sup&gt; (L); 0.17; 0.15</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>Sp7</td>
<td>2.5&lt;sup&gt;a&lt;/sup&gt;; 1.74&lt;sup&gt;a&lt;/sup&gt;; 0.81&lt;sup&gt;a&lt;/sup&gt; (L); 0.70 (L); 0.64&lt;sup&gt;a&lt;/sup&gt; (L); 0.21; 0.2</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>Cd</td>
<td>2.6&lt;sup&gt;a&lt;/sup&gt;; 1.77&lt;sup&gt;a&lt;/sup&gt;; 0.81&lt;sup&gt;a&lt;/sup&gt; (L); 0.71 (L); 0.64&lt;sup&gt;a&lt;/sup&gt; (L); 0.21; 0.19</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>Sp245</td>
<td>2.6&lt;sup&gt;a&lt;/sup&gt;; 1.76&lt;sup&gt;a&lt;/sup&gt;; 0.89&lt;sup&gt;a&lt;/sup&gt; (L); 0.78 (L); 0.72&lt;sup&gt;a&lt;/sup&gt; (L); 0.21; 0.14</td>
<td>7.1</td>
</tr>
<tr>
<td><em>A. lipoferum</em></td>
<td>Sp59b</td>
<td>2.6&lt;sup&gt;a&lt;/sup&gt;; 1.8&lt;sup&gt;a&lt;/sup&gt;; 1.38&lt;sup&gt;a&lt;/sup&gt;; 1.18&lt;sup&gt;a&lt;/sup&gt; (L); 0.97&lt;sup&gt;a&lt;/sup&gt; (L); 0.71 (L); 0.65&lt;sup&gt;a&lt;/sup&gt; (L); 0.4</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>JA25</td>
<td>2.25&lt;sup&gt;a&lt;/sup&gt;(ND); 1.8&lt;sup&gt;a&lt;/sup&gt;; 1.1&lt;sup&gt;a&lt;/sup&gt; (L); 0.85&lt;sup&gt;a&lt;/sup&gt; (L); 0.55&lt;sup&gt;a&lt;/sup&gt; (L); 0.45 (L); 0.3; 0.27; 0.22; 0.15</td>
<td>7.9</td>
</tr>
<tr>
<td><em>A. amazone</em>n<em>ens</em></td>
<td>Y2</td>
<td>2.7&lt;sup&gt;a&lt;/sup&gt;; 2.2; 1.7&lt;sup&gt;a&lt;/sup&gt;; 0.75</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>Y6</td>
<td>2.6&lt;sup&gt;a&lt;/sup&gt;; 2.1; 1.8&lt;sup&gt;a&lt;/sup&gt;; 0.71</td>
<td>7.2</td>
</tr>
<tr>
<td><em>A. irakense</em></td>
<td></td>
<td>2.4&lt;sup&gt;a&lt;/sup&gt;; 1.2&lt;sup&gt;a&lt;/sup&gt;; 0.95&lt;sup&gt;a&lt;/sup&gt;; 0.22</td>
<td>4.8</td>
</tr>
<tr>
<td><em>A. halopraeferens</em></td>
<td></td>
<td>2.6&lt;sup&gt;a&lt;/sup&gt;; 1.2&lt;sup&gt;a&lt;/sup&gt;; 0.98&lt;sup&gt;a&lt;/sup&gt;; 0.92&lt;sup&gt;a&lt;/sup&gt;; 0.22</td>
<td>5.9</td>
</tr>
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</table>

<sup>a</sup> Hybridized with 16S rDNA.

<sup>b</sup> Hybridized with nifHDK.

<sup>c</sup> The indicated molecular sizes are the averages of at least five determinations (except for those of *A. irakense* and *A. halopraeferens*, for which two independent experiments were performed), with a standard deviation of less than 10%. Chromosomes of *S. cerevisiae*, *Schizosaccharomyces pombe* or λ DNA concatemers (Amersham Pharmacia or Bio-Rad) were used as molecular size markers. The genome sizes were estimated based on those of the indicated replicons.

<sup>d</sup> Abbreviations: (L), Indication of linear molecule; ND, hybridization with 16S rDNA not determined.
Due to the high molecular weight of *Azospirillum* replicons, we reasoned that essential genes might be present in more than one replicon, indicating the presence of multiple chromosomes. DNA hybridization studies showed that all strains tested had at least two replicons hybridizing with a 16S rDNA probe from *A. brasilense* (Fig. 2 and Table 1). Wood et al. (31) reported the presence of minichromosomes in *A. brasilense* and *A. lipoferum*, based on the sizes of the DNA bands determined by vertical Eckhardt-type agarose gel electrophoresis; however, no localization or mapping of essential genes was reported. The DNA profile of Sp7 obtained by Wood et al. (31) was very similar to ours, with three bands in the region of 0.6 Mbp and two bands above 1.7 Mbp. Recently, Caballero-Mellado et al. (4) also analyzed several strains of *A. brasilense* by a horizontal Eckhardt-type gel electrophoresis and reported the presence of more than one replicon carrying 16S rDNA genes. However, these authors did not observe DNA hybridization with the largest replicon of strain Sp7 and suggested that this was probably due to its very high molecular weight (4). In addition, the genome profile of the Sp7 strain obtained by Caballero-Mellado et al. (4) was slightly different from that reported here and from that obtained by Wood et al. (31), a result probably due to electrophoresis resolution of the different experimental conditions.

While the 16S rRNA gene was found in several replicons in *Azospirillum*, the *nifHDK* operon seems to be located in only one replicon, at least for *A. brasilense* strains FP2 and Sp7. These genes were present in the largest replicon of these strains (Table 1), as revealed by DNA hybridization with an *A. brasilense* *nifHDK* probe. In the other strains, the *nifDK* genes were clearly located in a circular DNA molecule, since a very intense hybridization signal was observed in the gel wells in a PFGE of intact DNA (data not shown).

*Azospirillum* spp. have one of the most complex patterns of high-molecular-weight DNA among the α-Proteobacteria so far described. All five species analyzed showed multiple replicons, and the presence of 16S rDNA genes in several of them supports the suggestion that the *Azospirillum* genome is split into several chromosome-like structures. In *A. brasilense* and *A. lipoferum*, these structures were found in either linear or circular DNA, as has also been observed for other species within the alpha subgroup of the *Proteobacteria* (17).

The differences in the DNA patterns found among strains of the same species suggest that although stable, the genome structures of these organisms seem to evolve faster than the species differentiation. The mechanism underlying the development of these genome structures is unknown but probably involves genetic rearrangements between homologous DNA sequences shared by two or more replicons, as shown in *Brucella* (10). It may also involve horizontal DNA exchange.

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