

DNA restriction fingerprint analysis of the soil bacterium *Azospirillum*

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Total DNAs of 18 strains of *Azospirillum* from different sources and geographical areas were compared by restriction endonuclease pattern analysis. Fragments obtained with *Hind*III or *Bgl*II were separated by PAGE and stained with silver nitrate. Each strain possessed a unique and reproducible fingerprint with each enzyme, thereby facilitating strain recognition. UPGMA analysis recovered clusters of band patterns that were compared to the distribution of species within the genus *Azospirillum*.

Introduction

Bacteria of the genus *Azospirillum* are capable of nitrogen fixation under microaerophilic conditions. Interest in this genus has increased since Döbereiner & Day (1975) demonstrated its association with the roots of grasses, including important cereals like maize and wheat. Four species of *Azospirillum* have been described: *A. amazonense*, *A. brasilense*, *A. halopraeferens* and *A. lipoferum* (Krieg & Döbereiner, 1984; Reinhold *et al.*, 1987); these species were established on the basis of morphological, nutritional, physiological and biochemical characters as well as by determining the nucleic acid relatedness of many isolates.

One of the main objectives of research on *Azospirillum* is to obtain strains that could be used as biological fertilizers (Okon, 1985); for this reason and due to their widespread occurrence in tropical, temperate and cold soils (Döbereiner & Day, 1975; Haahtela *et al.*, 1981; Lamm & Neyra, 1981), hundreds of strains of azospirilla have been isolated in different countries. This raises the problem of their accurate identification; moreover, strains used as inoculants need to be traced and detected in soil in order to evaluate their effect on plant yield. Routine identification of *Azospirillum* usually depends on cultural and morphological criteria; however, these techniques do not allow strains to be distinguished from each other and sometimes clear attribution to a species is not easy.

In recent years, restriction endonuclease analysis of the genome has proved a useful tool in the differentiation of viruses (Skare *et al.*, 1975; Chowdhury *et al.*, 1986) and

bacteria (Mielenz *et al.*, 1979; Bjorvatn *et al.*, 1984; Dobritsa, 1985; Grothues & Tümmler, 1987). Results obtained so far with diverse genera indicate that this technique is highly reliable and easy to perform on a large number of isolates (Kristiansen *et al.*, 1985; Allardet-Servent *et al.*, 1988; Peterson & De La Maza, 1988). Few reports deal with computer-assisted numerical analysis of restriction fingerprints (Hookey *et al.*, 1985; Sorensen *et al.*, 1985). In the present work 18 strains of *Azospirillum*, representing the four established species and unclassified strains, were analysed.

Methods

Bacterial strains and growth conditions. The strains used, and their origins, are listed in Table 1. Strains of *Azospirillum brasilense* and *A. lipoferum* and incompletely identified strains from Italian soils were grown in Oxoid Antibiotic medium no. 3, strains of *A. amazonense* in Nutrient Broth (Difco) at pH 6.5 and *A. halopraeferens* Au5 in SM medium (Reinhold *et al.*, 1985) supplemented with 0.25% NaCl and adjusted to pH 7.2. Cultures were incubated at 35 °C (*Azospirillum* sp., *A. brasilense*, *A. lipoferum* and *A. amazonense*) or at 41 °C (*A. halopraeferens*) and shaken vigorously.

Preparation of total DNA. Cells were harvested in the early stationary phase, washed once with TEN buffer (10 mM-Tris/HCl, 1 mM-EDTA, 10 mM-NaCl, pH 8) and stored at -20 °C until needed. Organic solvents for cell extraction were prepared according to Maniatis *et al.* (1982). Cells suspended in TEN were lysed by adding SDS and proteinase K to final concentrations of 5 mg ml⁻¹ and 50 µg ml⁻¹ respectively. The NaCl concentration was raised to 300 mM and the lysate extracted twice with phenol/chloroform (1:1, v/v) and twice with chloroform. DNA was precipitated with 2 vols cold ethanol, collected with a glass rod and resuspended in TEN. RNA was

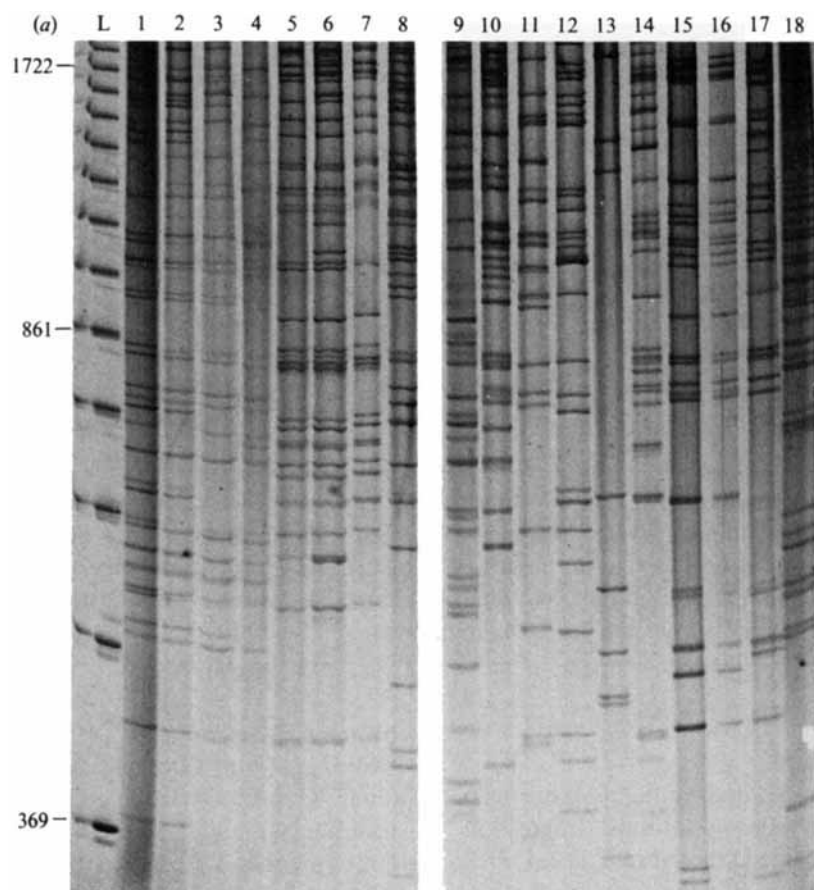


Fig. 1. (continued on facing page). SDS-PAGE profiles of *Azospirillum* total DNAs digested with restriction endonucleases *Hind*III (a) or *Bgl*II (b). The strains are designated by the reference numbers listed in Table 1. Lanes L contain BRL 123 bp DNA Ladder (a) or BRL 1 kb DNA Ladder (b).

Table 1. *Azospirillum* strains used

Ref.*	Species	Strain	Source	Received from †
5	<i>A. brasilense</i>	Sp6	<i>Tillandsia</i> , Italy	F. Favilli
6	<i>A. brasilense</i>	DSM 1690 ‡	<i>Digitaria</i> , Brazil	DSM
7	<i>A. brasilense</i>	Cd	<i>Cynodon dactylon</i> , USA	J. Döbereiner
10	<i>A. brasilense</i>	F14	Wheat, Italy	F. Favilli
14	<i>A. lipoferum</i>	Sp242	Maize, Brazil	J. Döbereiner
8	<i>A. lipoferum</i>	Col5	<i>Hyparrhenia rufa</i> , Colombia	
9	<i>A. lipoferum</i>	SpBr17	Maize, Brazil	
15	<i>A. amazonense</i>	DSM 2787	<i>Digitaria decumbens</i> , Brazil	DSM
16	<i>A. amazonense</i>	Y1	<i>Digitaria decumbens</i> , Brazil	A. Hartmann
17	<i>A. amazonense</i>	Y2	Unknown, Brazil	
18	<i>A. amazonense</i>	Y6	<i>Pennisetum purpureum</i> , Brazil	
13	<i>A. halopraeferens</i>	Au5	<i>Leptochloa fusca</i> , Pakistan	F. Favilli
1	<i>Azospirillum</i> sp. §	T1	Tobacco, Italy	
2	<i>Azospirillum</i> sp. §	T2	Tobacco, Italy	
3	<i>Azospirillum</i> sp. §	T5	Tobacco, Italy	F. Favilli
4	<i>Azospirillum</i> sp. §	T8	Tobacco, Italy	
11	<i>Azospirillum</i> sp. §	M6	Maize, Italy	
12	<i>Azospirillum</i> sp. §	M1	Maize, Italy	

* Reference number in Fig. 1.

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‡ This strain is the same as strain Sp7 isolated by J. Döbereiner.

§ These strains could not be assigned to any of the four described species by following species descriptions (Krieg & Döbereiner, 1984; Reinhold et al., 1987) and the recommendations of Döbereiner (1987) and Döbereiner & Pedrosa (1987) for the identification of azospirilla.

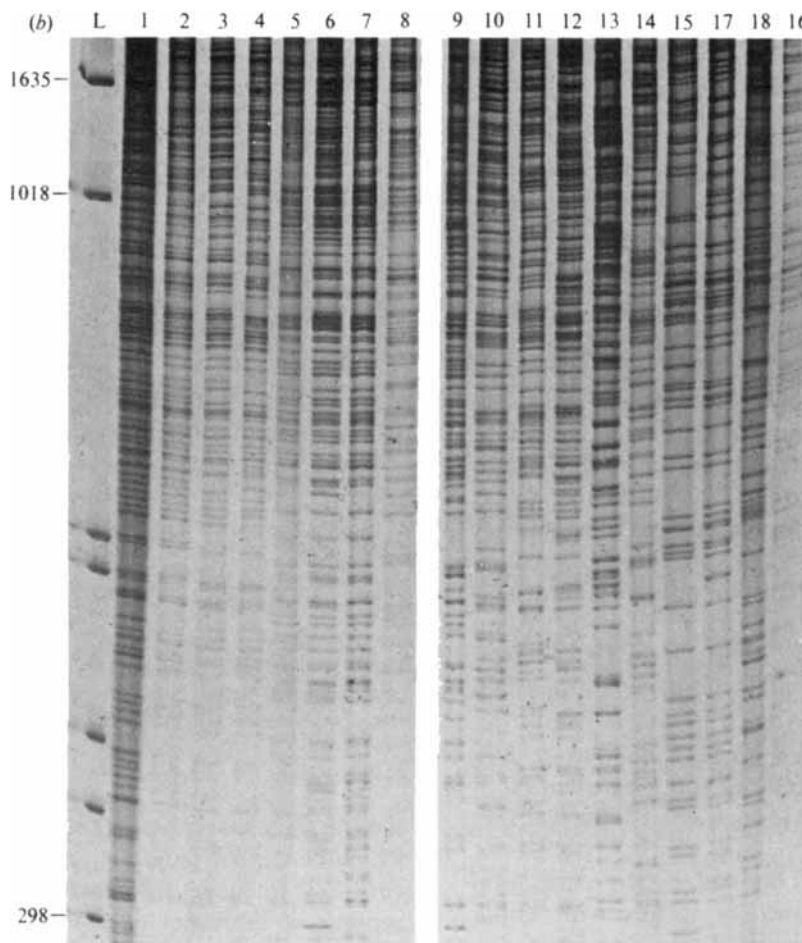


Fig. 1 (contd.)

hydrolysed with 50 µg RNAase A ml⁻¹ and 3 units RNAase T₁ ml⁻¹. The DNA was then extracted once with phenol/chloroform (1:1, v/v) and twice with chloroform, precipitated with cold ethanol, collected with a glass rod and resuspended in TE buffer (10 mM-Tris/HCl, 1 mM-EDTA, pH 8). DNA purity and concentration were checked spectrophotometrically following Maniatis *et al.* (1982).

Digestion of DNA. Restriction endonucleases were purchased from Boehringer Mannheim Biochemicals. Ten micrograms of DNA were completely digested with 20 units of enzyme by incubating the mixture at 37 °C overnight, as recommended by the supplier.

SDS-PAGE. DNA digests were loaded on a vertical discontinuous 7.5% (w/v) acrylamide gel according to Laemmli (1970) and run at 17 mA for 17 h (19 h when running *Hind*III fragments). BRL 1 kb DNA Ladder or BRL 123 bp DNA Ladder were added as markers. The running gel was 22 cm long and 1.5 mm thick.

Gels were fixed and washed by soaking and gently shaking for 30 min in each of the following solutions: 50% ethanol/10% acetic acid; 25% ethanol/10% acetic acid; 10% ethanol/0.5% acetic acid (all % values are v/v). They were then shaken for 2 h in silver nitrate solution (1.9 g l⁻¹), rinsed with distilled water and developed with formaldehyde (7.5 g l⁻¹) in 0.75 M-sodium hydroxide until the bands reached the desired density. Better contrast was achieved by treating the gels for 1 h with sodium carbonate (7.5 g l⁻¹).

Restriction pattern analysis. Silver-stained gels were scanned with an LKB Ultrosan II laser densitometer connected to an Olivetti M240 personal computer. Integration and peak detection were performed with LKB 2400 GelScan XL program. Only band positions were taken into account, irrespective of any difference in density. Each band was defined by its starting and ending points and its peak position given by the INTEGRATE procedure with the GAUSSIAN and VALLEY options of the 2400 GelScan XL program. A dedicated program written in dBASE IV was used to rescale the peak positions, to compare band patterns and to calculate similarity coefficients. Two distant evident homologous bands present in each lane were used as internal standards to correct for the 'smiling' effect: common values were given to each set of homologous band peaks and the integration results of each trace were rescaled on these new values. The number of bands at coincident positions was obtained from pairwise comparisons of rescaled traces: when a peak value of a band in a trace was included between the values of the starting and ending points of a band in the other trace, then those two bands were taken to have coincident positions (i.e. to be homologous). A tolerance value (0.1% of running gel length) was applied to either side of each band to compensate for misalignment of homologous bands due to technical imperfections (Hood *et al.*, 1988). Similarity between each pair of strains was calculated as the Dice coefficient, S_D : the ratio of twice the number of bands common to their patterns, to the sum of all bands in the two patterns. Strains were clustered by the UPGMA method (Sneath & Sokal, 1973) using the CLUSTER and TREE procedures of the SAS package (SAS Institute Inc., 1987).

Results and Discussion

Choice of restriction endonucleases and reproducibility of the method

DNA of 18 strains of *Azospirillum* from different sources and geographical areas (Table 1) was analysed by SDS-PAGE of the fragments obtained after digestion of total DNA with restriction endonucleases. SDS-PAGE was chosen as the separation technique because it offered better resolution than agarose gel electrophoresis, thereby enabling sharp separation of the low-molecular-mass nucleic acid fragments. Fragments were stained using the highly sensitive silver nitrate method (Goldman & Merrill, 1982). Digestion of total bacterial DNA with restriction endonucleases that recognize 6 bp sites provides a wide range of fragment sizes. The smaller fragments, between 300 and 1700 bp, that were readily separated with the highest resolution, were chosen as representative fingerprints of each bacterial strain. Restriction endonucleases *Ban*II, *Hind*III, *Eco*RI, *Eco*RV, *Bgl*II, *Dra*I, *Hpa*I, *Kpn*I, *Pst*I, *Xba*I and *Cla*I were tested with total DNA of *A. brasilense* strain Cd. Endonucleases *Hind*III and *Bgl*II were found to yield, in the selected size range, a number and a distribution of fragments suitable for reliable analysis with the GelScan program.

The electropherograms obtained by digesting total DNA from the 18 *Azospirillum* strains with either *Hind*III or *Bgl*II are shown in Fig. 1(a) and (b), respectively. These show that, with both enzymes, each strain possesses a unique restriction pattern. In replicate experiments, using DNA obtained in separate extractions, each of the patterns was shown to be completely reproducible. The type strain of *A. amazonense*, obtained from two sources, A. Hartmann (strain Y1; ref. no. 16) and the DSM (DSM 2787; ref. no 15) maintained the same pattern even after repeated subculturing in separate laboratories (Fig. 1a, b). A spontaneous rifampicin-resistant mutant of *A. brasilense* Sp6 remained unaltered in its banding pattern (data not shown). The restriction pattern of total DNA thus constituted a unique fingerprint of each organism and was considered suitable for the reliable recognition of strains.

Restriction pattern analysis

Digestion with *Hind*III gave fewer bands than digestion with *Bgl*II. The gel with *Hind*III digests was analysed by scanning densitometry in the region from 370 to 1700 bp and the gel with *Bgl*II digests from 300 to 1300 bp. The selected intervals contained between 47 and 92 DNA bands per strain in the *Bgl*II gel, and between 24 and 36 bands per strain in the *Hind*III gel. The exception was

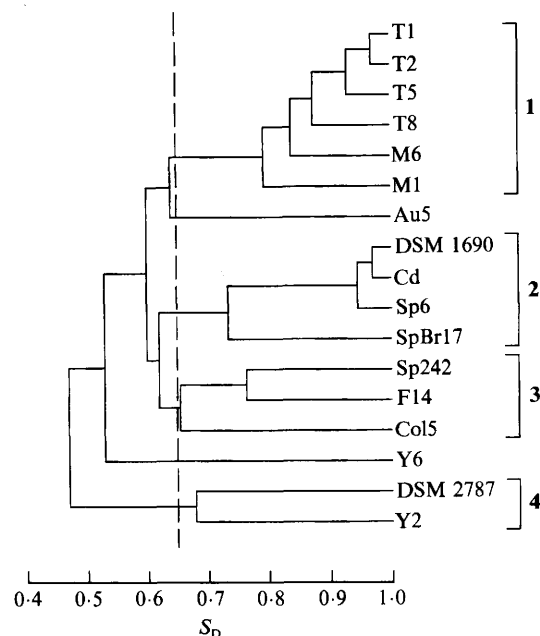


Fig. 2. Dendrogram based on UPGMA clustering of the S_D matrix obtained from *Bgl*II restriction pattern analysis. Square brackets identify the clusters obtained with a cut-off value of 0.65 (dashed line).

the *Hind*III electropherogram of strain Au5 (ref. no. 13), which contained only eight bands, indicating either a very low frequency of *Hind*III restriction sites in its genome, or inhibition of hydrolysis due to methylation. The latter occurrence would limit the usefulness of the method for the study of the relationship among strains, but should not interfere with the identification of a single strain. The matrix of S_D coefficients obtained from the analyses of the two sets of restriction patterns showed lower values for *Hind*III digestions than for *Bgl*II digestions (not shown). This difference, which cannot be easily explained, indicates that the calculated S_D between strains is affected by the choice of the restriction enzyme. This suggests that caution is required in the screening and in the selection of the restriction enzyme in order to avoid misinterpretation of data. However, in the present case, UPGMA clustering (see below) applied to both sets of data gave almost correspondent groupings (dendrogram of *Hind*III matrix not shown). Fig. 2 shows the resultant UPGMA dendrogram of the *Bgl*II matrix. Using an S_D cut-off value of 0.65, four aggregate clusters plus two single-member clusters were recovered. Cluster 1 contains the strains isolated in Italy from tobacco and maize. The restriction fingerprint method does not allow these strains to be assigned to the four described species of *Azospirillum*. However, this was not surprising since these strains could not be identified using established tests (Döbereiner & Pedrosa, 1987). Cluster 2 contains three strains of *A. brasilense* and *A. lipoferum* SpBr17.

The *A. brasilense* strains cluster close together despite originating from different countries. Cluster 3 comprises *A. brasilense* F14 and two strains of *A. lipoferum*; however, one of the *A. lipoferum* strains (Col 5) joins the cluster near to the cut-off value. A clear separation between *A. lipoferum* and *A. brasilense* cannot be inferred from this study because strains belonging to these two species are mixed in clusters 2 and 3. This was partly expected because *A. lipoferum* strains Sp242 and SpBr17 are rather atypical (Döbereiner, 1983; J. Döbereiner, personal communication), and DNA:DNA hybridization values among strains of *A. lipoferum* are low (Falk *et al.*, 1986). Cluster 4, comprising *A. amazonense*, strains DSM 2787 and Y2, shows an S_D value lower than 0.50 with all other strains, including *A. amazonense* Y6, which formed a single-member cluster. These data support the suggestion that *A. amazonense* is heterogeneous, and distant from the other azospirilla (Falk *et al.*, 1986; Reinhold *et al.*, 1987; Schenk & Werner, 1988). Strain Au5, which was the only representative of the species *A. halopraeferens* analysed, also formed a single-member cluster, although it has an S_D value of 0.64 with cluster 1.

Our results indicate that DNA restriction pattern analysis enables bacteria of the genus *Azospirillum* belonging to the same or to closely related species to be differentiated. This approach may be valuable for the identification of re-isolated soil inoculants, screening of new isolates, and patenting of engineered strains.

Although few taxonomic conclusions can be drawn, our data indicate that total DNA restriction fingerprints could be used, in addition to routine identification tests, in the species attribution of isolates of *Azospirillum*, since, in most instances, an S_D of 0.65 separates clusters at the species level.

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