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AZOSPIRILLUM/PLANT ASSOCIATIONS

Edited by
Yaacov Okon

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PREFACE

Free living nitrogen-fixing azospirilla exert beneficial effects on plant growth and yield of many crops of agronomic importance, such as grains, forage grasses, legumes and tomatoes. This has been demonstrated in many field inoculation experiments carried out during the past 18 years in different locations. The fact that companies are developing commercial *Azospirillum* inoculants is a very important achievement. Their economical impact in promoting crop yields and a more efficient utilization of fertilizer is now being evaluated the world over.

As seen in this book, bacteria of the Genus *Azospirillum* are, from a physiological and molecular point of view, extremely interesting soil microorganisms. They are very versatile in their carbon and nitrogen metabolism. The ecology of their associations in the rhizosphere and the physiological and morphological effects on inoculated roots constitute a unique model for studying plant-bacterial interactions.

This book presents state of the art information about *Azospirillum*. It covers, in an interdisciplinary manner, the taxonomy, physiology, and genetics of *Azospirillum* (Chapters 1, 2, and 3) and the rhizosphere ecology, effects on plant growth, agronomic utilization, and positive interactions of *Azospirillum* with other beneficial soil microorganisms (Chapters 4, 5, 6, and 7).

In Chapter 8, an up-to-date evaluation is presented, giving the potential of biological nitrogen fixation by several species of nitrogen-fixing bacteria in association with grasses. In Chapter 9, the utilization of other plant growth-promoting rhizobacteria (PGPR) adds the perspective of a broader field of investigation.

I would like to express my appreciation and gratitude to the collaborating authors for sharing their research experience and knowledge of *Azospirillum*, associative biological nitrogen fixation, and PGPR.

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THE EDITOR

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Dr. Okon received his B.S., M.S., and Ph.D. degrees in 1967, 1970, and 1974, respectively, from the Department of Plant Pathology and Microbiology, Faculty of Agriculture, The Hebrew University of Jerusalem. After doing post-doctoral work on biological nitrogen fixation at the Department of Biochemistry, College of Agriculture, University of Wisconsin–Madison in Dr. R. H. Burris' laboratory, Dr. Okon was appointed Lecturer of Agricultural Microbiology at The Hebrew University of Jerusalem, Rehovot. He became Senior Lecturer in 1979, Associate Professor in 1982, and Professor in 1986.

Dr. Okon is a member of the Israeli Society for Microbiology and the Israel Phytopathological Society. He is also a member of the editorial board of Arid Soil Research and Rehabilitation. In 1980–1981 he was a visiting scientist at the Central Research and Development Department, Experimental Station, E.I. du Pont de Nemours & Co., Wilmington, Delaware, and in 1987–1988 at the Department of Biotechnology, Pasteur Institute, Paris, France.

Dr. Okon has presented over 20 invited lectures at international meetings, given approximately 50 guest lectures at various universities and institutes, and has taught invited courses on plant-bacterial interactions in Mexico, Uruguay, Argentina, Italy, and Germany. He has published more than 130 research papers and has been the recipient of research grants from the U.S.-Israel Binational Agricultural Research and Development Fund (BARD), the U.S.-Israel Binational Science Foundation (BSF), the Israel Ministry of Agriculture, the Israel Academy of Sciences, and from various biotechnology companies. His current research interests are in elucidating the mechanisms of plant growth promotion by *Azospirillum* and its application for the benefit of agriculture.

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CONTENTS

Chapter 1

Taxonomy of *Azospirillum*

Monique Gillis and Barbara Reinhold-Hurek

CONTENTS

I. INTRODUCTION

In early microbiology, classification of bacteria was based on some striking morphological features and a few physiological characteristics. It is obvious that such a classification relies only on a small part of the bacterial genome and consequently yields insufficient information. During the last 15 years, a wide variety of new techniques were developed to perform comparative studies on various aspects of the bacterial cell. Several have now become standard techniques in bacterial taxonomy. In the last years, it became more and more evident that stable conclusions on the different levels of classification that reflect the natural relationships could only be obtained by using polyphasic taxonomy.

Generally speaking, the polyphasic approach can be described as the study of any group of bacteria using a set of techniques chosen for its particular taxonomic relevance. In bacteria, different levels of information can be studied. The total DNA is, of course, the primary level of information. A second level is the structure of the proteins. A third level of expression is contained in the chemical structure of cellular components while the phenotypic behavior can be considered as a fourth level.

The total DNA can be characterized by the following parameters: mean percent (G+C), genome size, degree of hybridization, transformation capability, patterns obtained after restriction endonuclease digestion, followed by agarose gel electrophoresis of the fragments (restriction fragment length polymorphism, or RFLP).[20,23-25,50] Parts of the genome are currently studied by RFLPs in combination with hybridization with specific DNA probes;[20,26,29] by comparison of the plasmid profiles,[26,57] by RFLPs of the plasmids,[26] by RFLPs of specifically amplified parts of the genome,[55] and by amplification using primers corresponding to repetitive sequences.[9] Conserved molecules such as 23S, 16S, and 5S rRNA have been studied intensively[11,14,62,63] because of their relevance for differentiating genera, families, subclasses, etc; they have been studied by cataloguing and sequencing,[62,63] and by rRNA:DNA hybridizations.[11] Nowadays rRNA parameters are indispensable in classification, identification, and description of new taxa.[28,41] Since different new, fast techniques became available to sequence rRNA,[37] or to regenerate and sequence rDNA (total or parts of it),[2,5,37,60,66] 16S rDNA sequences are used more and more to characterize bacterial groups (genera and in some cases species), at the same time, the use of the 5S rRNA sequences has decreased. Moreover, to identify closely related groups, 23S rDNA[35] and the intercistronic sequences are also being used now, because the hypervariable parts do allow a very fine characterization (species and even subspecies level).[55]

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For comparative purposes, proteins can be studied by amino acid sequencing, one[32,44]- or two[12]-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the cellular proteins, zymograms, multilocus enzyme electrophoresis,[40,48] and various immunological techniques.

The third level of expression is contained in the structure of particular cell constituents that are mostly referred to as chemotaxonomic markers. Several of these markers are well-known objects of study, e.g., cell wall compounds, the quinones, exopolysaccharides, lipopolysaccharides, and polyamines.[6] Special attention is paid to lipids and particularly to fatty acids. The gas-chromatographic analysis of methylated cellular fatty acids (fatty acid methyl esters or FAMEs) became a widespread identification technique that can also be used to unravel fine relationships between large groups of isolates.[56,58,64]

The last level of information can be studied by phenotypical analysis including morphological and a wide variety of physiological, enzymatic, and nutritional features. Diverse commercialized systems, e.g., from API (BioMérieux, France) and BIOLOG (Biolog Corporation, Hayward, California, U.S.A.) can be used. Computer-assisted methods are needed to analyze the results because the inclusion of most of the available strains is an essential point in this methodology.

The described techniques have different discriminating powers; some allow differentiation on a very fine level (subspecies, strain fingerprint), while others can be relevant in a broad taxonomic range. It is well accepted that in a polyphasic approach, the selection of the methods is very important and has to be made in proportion with the level of relationship that is being studied. As a rule, unknown isolates were first screened for groups of similar strains by a fast technique such as comparison of the FAME fingerprints, SDS-PAGE of the total bacterial proteins, multilocus enzyme electrophoresis, RFLPs of total DNA, or another technique with a fine differentiating power. Representatives of the different groups can then be included in subsequent genotypic characterization, including studies of the rRNA and DNA:DNA hybridization. The latter technique is very important to delineate species and the DNA relatedness is nowadays indispensable to distinguish and to describe new species.[28,41]

II. PHYLOGENETIC POSITION OF THE GENUS *AZOSPIRILLUM*

Because rRNA is an appropriate parameter to measure intra- and intergeneric (and even deeper) relationships it has been used extensively for that purpose. Within the domain of the *Bacteria*[61] several lineages have been detected of which a diverse group of Gram negative bacteria showed a tree-like deep evolutionary relationship. This group was given class status as the *Proteobacteria*.[51] By using rRNA:DNA hybridizations as well as by cataloguing and sequencing, analogous results were obtained: the class *Proteobacteria* consist of at least six large subgroups that have been named rRNA superfamilies or subclasses.[10,11,51] Nitrogen fixing bacteria have been found within at least six rRNA superfamilies.[11,65]

In a first approach it was shown by rRNA:DNA hybridizations and cataloguing of the 16S rRNA that *Azospirillum brasilense* belongs in group 1 of the alpha-subclass of the *Proteobacteria*[63] and that *Azospirillum lipoferum* (the only other species in *Azospirillum* at that time) has very similar rRNA cistrons and constitutes a separate rRNA branch* in rRNA superfamily IV[13] corresponding with the alpha-subclass of the *Proteobacteria*. Within rRNA superfamily IV this branch is part of an rRNA cluster consisting of three other rRNA branches containing respectively *Aquaspirillum polymorphum*, *Aquaspirillum*

* An rRNA branch consists of strains that have at most a difference of $T_{m(e)}$ of 12°C vs. the $T_{m(e)}$ of the homologous duplex; $T_{m(e)}$ is the melting temperature of an rRNA:DNA hybrid determined in standard conditions.[13]

perigrinum (both subspecies), and *Aquaspirillum itersonii* (subsp. *itersonii* and *vulgatum*) and the genus *Rhodospirillum*.[43] This rRNA cluster corresponds with the alpha-1 subgroup also containing *Rhodopseudomonas globiformis*[62] and *Magnetospirillum*.[49] These organisms are phylogenetically the closest relatives of *Azospirillum*. Because the different branches of this rRNA cluster or rRNA group split off rather low (at a $T_{m(c)}$ difference of approximately 10°C or at an overall sequence similarity of 84.1 to 88.9%[49]) these branches are not related at genus level.

Later, the species *Azospirillum amazonense* was described phenotypically,[38] together with a fourth group of N_2-fixing spirillae for which the name *"Azospirillum seropedicae"* was proposed.[18] rRNA:DNA competition experiments[19] demonstrated that *Azospirillum amazonense* is a relative of *Azospirillum brasilense* and *Azospirillum lipoferum*. *"Azospirillum seropedicae"* could not be retained in *Azospirillum* because analogous rRNA:DNA hybridization experiments indicated that these organisms belonged in another subclass of the *Proteobacteria* and consequently a new genus *Herbaspirillum* with one species *Herbaspirillum seropedicae* has been proposed.[1] Later, it was shown that the latter group constitutes a separate rRNA branch in rRNA superfamily III together with *[Pseudomonas]* rubrisubalbicans*, some isolates from clinical origin, and *[Aquaspirillum]* autorophicum*.[21] The species *Azospirillum amazonense* was also shown to be a member of the *Azospirillum* rRNA branch in rRNA superfamily IV[47] but in a lower position ($T_{m(c)}$ of 73.4 to 75.3°C) clearly demonstrating that it is phylogenetically more distinct from the species *Azospirillum brasilense* and *Azospirillum lipoferum* that both have $T_{m(c)}$ values of 80 to 82.5°C. The close relationship between *Azospirillum brasilense* and *Azospirillum lipoferum* has been confirmed by DNA:DNA hybridizations.[52]

Later, a new group of diazotrophs associated with the roots of *Leptochloa fusca* (L) Kunth, grown in salt affected soils in the Punjab region of Pakistan, was found and described as having a phenotypic resemblance with the known *Azospirillum* species although they could not be assigned to any of these species. Here again rRNA:DNA hybridizations with labeled rRNA from *Azospirillum brasilense* ATCC 29145^T clearly demonstrated that this group of diazotrophs was also a member of the *Azospirillum* rRNA branch; their $T_{m(c)}$ values (74.0 to 74.8°C) had the same range as *Azospirillum amazonense*. The combination of the results obtained by other techniques led to the conclusion that this group constituted a fourth species: *Azospirillum halopraeferens*.[47]

In 1990, a new group of bacteria with the overall properties of *Azospirillum* and containing seven strains was described; they were isolated from the roots and rhizosphere of rice in the region of Diwaniyah (Quadisya) in Iraq[33] and could not be identified as one of the known species. Because within *Azospirillum* they constitute a single DNA homology group corresponding with a separate phenotypic cluster, a new species *Azospirillum irakense* has been proposed[33] and described. Direct sequencing of a polymerase chain reaction (PCR)-amplified 16S rDNA gene[2] located this species on the same branch as *Azospirillum lipoferum*, indicating that it does also genotypically belong in *Azospirillum*. The closest related taxa to *Azospirillum irakense* and *Azospirillum lipoferum* are *Magnetospirillum magnetotacticum* and *Rhodospirillum rubrum*;[2] other representatives of the alpha-subclass have been included in this sequencing study and have indeed been found to belong in another group (see Figure 2). Other sequencing results[8] confirm that *Aquaspirillum itersonii* and *Aquaspirillum polymorphum* constitute one group with *Magnetospirillum magnetotacticum* and suggest a closer relationship of the latter with *Aquaspirillum polymorphum* than with *Aquaspirillum itersonii*. The correspondence with the dendrogram in Figure 1 is good as could be expected for two techniques investigating the same

*Brackets are used to indicate strains that do not belong in the genus on the base of phylogenetic data.

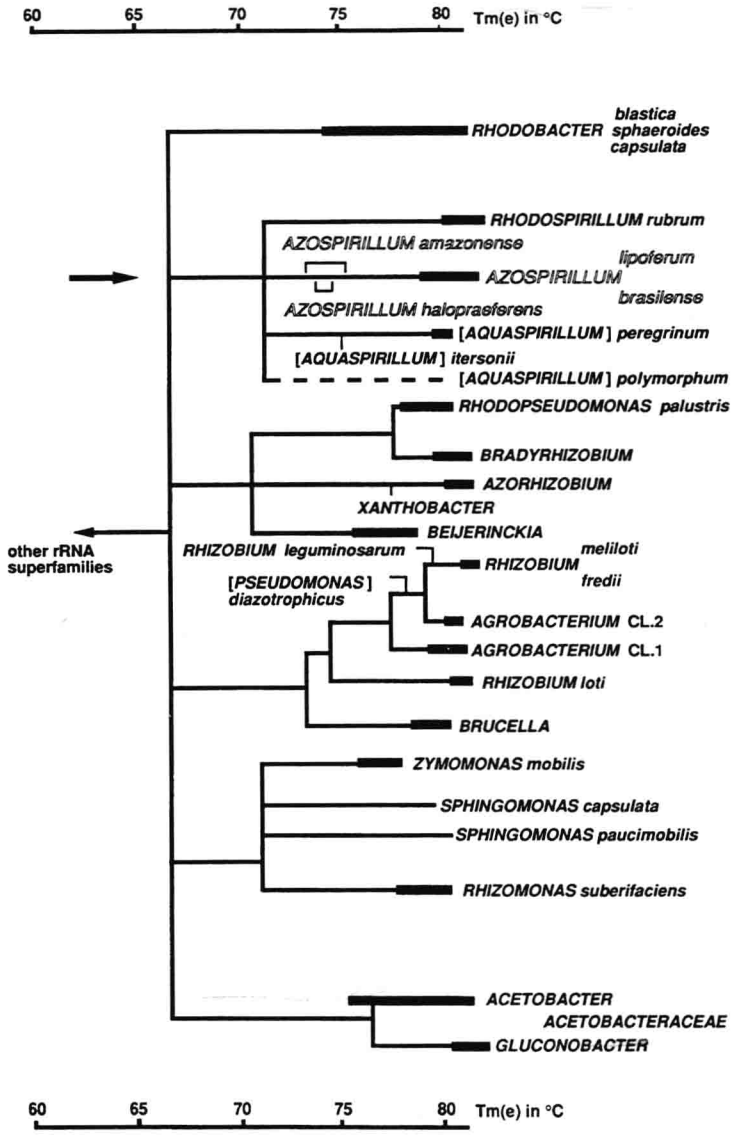

Figure 1. Dendrogram, showing the phylogenetic relationships of the genus *Azospirillum* with the other bacterial taxa of rRNA superfamily IV. All data are expressed as $T_{m(e)}$ in degrees Celsius. The different rRNA branches are given; solid bars represent the $T_{m(e)}$ range of the rRNA branch.

macromolecule. Nevertheless, up to now it is not clear whether *Azospirillum irakense* is genotypically closer to *Azospirillum amazonence* and *Azospirillum halopraeferens* than to *Azospirillum brasilense* and *Azospirillum lipoferum*. According to the partial sequence of its 16S rRNA, *Azospirillum irakense* has a similarity of 87% with that of *Azospirillum lipoferum*[2] that is rather low when compared with the results of Schleifer et al.[49] They found a similarity of 84.1 to 88.9 between *Magnetospirillum* and the reference organisms of the

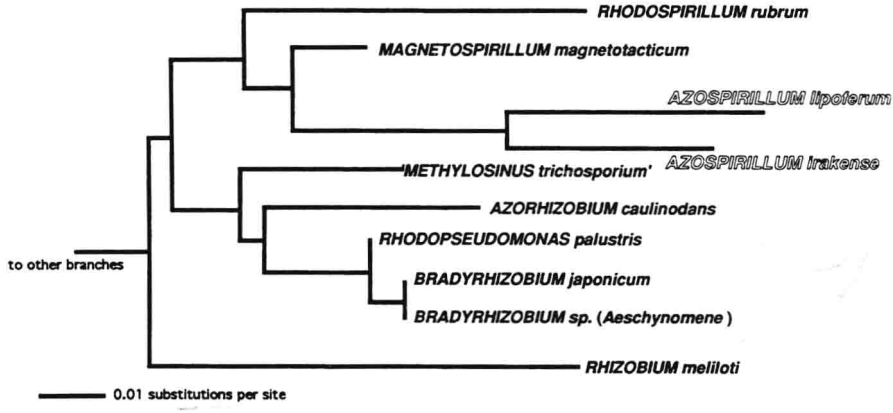

Figure 2. Tree constructed with partial 16S sequences (51–232) of *Azospirillum irakense, A. lipoferum* and some other representatives of subclass alpha group-1. (From Bally, R. Simonet, P., Haurat, J., and Normand, P., *Symbiosis,* 13, 47, 1992. With permission.)

alpha subclass. It can be concluded that the genus *Azospirillum* constitute phylogenetically a separate entity in which *Azospirillum amazonense* and *Azospirillum halopraeferens* and probably also *Azospirillum irakense* are the most divergent from *Azospirillum brasilense* and *Azospirillum lipoferum.*

III. DIFFERENTIATION FROM OTHER DIAZOTROPHIC GENERA

Diazotrophic bacteria are scattered over the different rRNA superfamilies.[11] The phenotypic differentiation of azospirilla from diazotrophic bacteria belonging in other rRNA superfamilies than rRNA superfamily IV, is mainly based on the vibroid shape of the azospirilla, the cell diameter, the type of flagellation, the nitrogen fixation that occurs only under microaerophilic conditions and the percent G+C.[15,36] Of course partial rRNA sequences, rRNA:DNA hybridizations, and chemotaxonomic markers such as isoprenoid quinones[7] and polyamines[6] can also be used to determine in which rRNA superfamily or subclass an unknown isolate belongs. The differentiation from *Herbaspirillum seropedicae* can be made by the type of flagellation and the smaller diameter of the *Herbaspirillum* cell.[15]

The highest concentration of diazotrophs was found within rRNA superfamily IV. *Azospirillum, Rhodospirillum, [Aquaspirillum] itersonii* and *[Aquaspirillum] perigrinum* and *Magnetospirillum* are members of one rRNA cluster. Other diazotrophic groups as *Rhodomicrobium, Rhodopila, Rhodobacter, Rhodopseudomonas, Bradyrhizobium, Azorhizobium, Xanthobacter, Beijerinckia, Rhizobium, Ancylobacter, Acetobacter diazotrophicus, Mycoplana* and two species of obligate methylotrophic bacteria[16,22,31,43,45,53,59,62,63] constitute within this rRNA superfamily separate rRNA branches or rRNA clusters (that are related with *Azospirillum* at the base $T_{m(e)}$ level of this rRNA superfamily which is approximately 67°C). A diazotrophic isolate from rice[3] has been identified as *[Pseudomonas] paucimobilis* and belongs in the *Sphingomonas paucimobilis* rRNA branch.[54,64] *[Pseudomonas] diazotrophicus* isolated from the roots of wetland rice[58] is a member of the *Rhizobium-Agrobacterium* rRNA cluster.[11]

Azospirillum can be differentiated from the members of the *Rhodospirillaceae* by the lack of phototrophy, and from *Rhizobium, Bradyrhizobium,* and *Azorhizobium* by the impossibility to form root or stem hypertrophies. It differs from *Acetobacter diazotrophicus* in cell shape, the oxidation of acetic acid and lactic acid to CO_2 and percent G+C; the

Table 1 Differential Characteristics[a] of the Genus *Azospirillum* and Other Diazotrophic Groups from rRNA Superfamily IV

Characteristic	*Azospirillum*	*Xanthobacter*	*Beijerinckia*	*[Aquaspirillum] perigrinum*	*[Aquaspirillum] itersonii*[b]	*Magnetospirillum*	Strain 5AJ[c]	*[Pseudomonas] diazotrophicus*
Mainly a vibroid shape	+	–	–	H	H	H	–	–
Gram variability may occur	+	+	–	–	–	–	–	–
Motility	+[d]	+[g]	D	+	+	+	+	+
Flagellar arrangement								
Monotrichous	+[e]	–	–	–	–	–	+	+
Lophotrichous	–	–	–	BT	BT	BS	–	
Peritrichous	–[e]	+	+	–	–	–	–	
Nitrogen fixed under microaerophilic conditions	+	+	–	+	+	+[f]	+	+
Some species or strains are denitrifiers	+	–	–	–	+	+[f]	–	–
Fermentative ability present	D	–	–	W	W	ND	–	–
Sucrose can be used as sole source	–	+	+	–	–	–	+	+
Microaerophilic	–	–	–	–	–	+	–	–
Magnetotactic	–	–	–	–	–	+	–	–
Mol% (G+C) of DNA	64–71	65–70	55–61	60–62	60–64	64–71	67.4	65–67

Note: Symbols: +, positive for all strains; –, negative for all strains; D, differs among strains; H, helical (one or more complete turns or twists); BT, bipolar tufts; BS, bipolar single flagellum; w, weak; ND, not determined.

[a] Results taken from References 3, 15, 22, 36, 39, 43, 45, 49, 58, and 59.

[b] One strain of *[Aquaspirillum] itersonii* subsp. *nipponicum* has been studied and did not belong in rRNA superfamily IV.

[c] This isolate was identified phenotypically as *Sphingomonas paucimobilis*.[3]

[d] One nonmotile *Azospirillum lipoferum* strain has been described.[3]

[e] In liquid medium, the cells possess one single polar flagellum; on agar media, lateral flagella also occur.

[f] Only known for the species *Magnetospirillum magnetotacticum*.[39]

[g] For some species, motility depends on growing conditions.[45]

methylotrophic diazotrophic members of rRNA superfamily IV are obligate methylotrophs. The features that discriminate *Azospirillum* from most of the other diazotrophic groups of rRNA superfamily IV are given in Table 1.

IV. DESCRIPTION AND IDENTIFICATION OF THE GENUS

Only the general characteristics are given; further descriptive information can be found in the literature.[15,33,36,47]

Azospirillum species are unable to initiate N_2-dependent growth on agar plates under air or in liquid medium, but do fix N_2 in N-free semisolid medium.[15] They are plump, slightly-curved and straight rods of 0.7 to 1.2 μm in diameter and 2.0 to 3.0 μm in length depending on the culture conditions. The cells are motile with a single polar flagellum (except an isolate of *Azospirillum lipoferum* isolated from rice[3]); on agar media, numerous lateral flagella can be formed. Cells are Gram negative to Gram variable. Intracellular poly-β-hydroxybutyrate granules are present. They possess mainly a respiratory type of metabolism with oxygen or nitrate as the terminal electron acceptor; weak fermentative ability has been observed with *Azospirillum lipoferum* grown on glucose or fructose. Optimum temperature is 33 to 41°C. Oxidase is positive. They grow well on the salts of organic acids such as malate, succinate, lactate, or pyruvate. Some strains require biotin. Some occur free living in the soil or associated with the roots of cereals, grasses, vegetables, legumes and tuber plants. The percent G+C ranges from 64 to 71%. The type species is *Azospirillum lipoferum* (Beijerinck, 1925).

V. CHARACTERISTICS OF THE DIFFERENT SPECIES

From the five species that have been described, *Azospirillum brasilense* and *Azospirillum lipoferum* have been isolated all over the world from soil and the roots of a variety of grasses and cereals. Other plants that harbor both species have been described,[15] and, recently, roots of sunflower have been added.[17] Both *Azospirillum* species have been studied intensively, and a large variety of features to differentiate them are available.[36] Two dimensional SDS-PAGE of total proteins can be used as a fingerprinting technique and to distinguish both species.[12] RFLPs of their total DNAs are proposed as a tool for strain identification;[20,24,25] with this technique *Azospirillum lipoferum* seems more heterogeneous than *Azospirillum brasilense*.[20] Both species differ also in the composition and RFLPs of their plasmids; a large 150 MDa plasmid is found in all *Azospirillum lipoferum* strains.[26] Some strains do produce melanin and there is laccase activity.[27,42] A weak DNA:DNA homology between total DNA of both species has been found.[33,52] Both species have similar FAME patterns,[58] 18:1 being the predominant nonhydroxy fatty acid.

Azospirillum amazonense was originally isolated from roots of forage grasses and certain palm trees in Brazil[38] but has later been found on other plant roots and in other regions. The strains are genotypically and phenotypically very similar.[33,47] RFLPs of their total DNA confirm their separate position.[24] The FAME pattern of this species is different from that of *Azospirillum brasilense* and *Azospirillum lipoferum;* it contains less 18:1 and more 16:0 nonhydroxy fatty acids.[58]

Up to now *Azospirillum halopraeferens* has only been isolated from the root surface of *Leptochloa fusca* in the Punjab (Pakistan); no *Azospirillum halopraeferens* has been identified among various azospirillae isolated from roots of various plants growing on saline soils in Brazil.[46] The different isolates are very similar.[47] Other isolates from Kallar grass have not yet been identified.[4]

Azospirillum irakense has only been found associated with the roots and the rhizosphere of rice in the region of Diwaniyah (Qadisya) in Iraq. A typical feature is the hydrolysis of

Table 2 Characteristics Differentiating the Five *Azospirillum* Species[a]

Feature	*A. lipoferum*	*A. brasilense*	*A. amazonense*	*A. halopraeferens*	*A. irakense*
Cell width (in μm)	1.0–1.7	1.0–1.2	0.9–1.0	0.7–1.4	0.6–0.9
Cell length exceeding 5 μm in alkaline N-free semisolid medium	+	–	–	–	+
Lateral flagella present	+	+	–	–	+
Optimum growth temperature	37	37	35	41	33
Growth and N_2-fixation at					
pH 7.5	+	+	–	+	+
pH 6.0	+	+	+	–	+
Growth with 3% NaCl	–	d	–	+	+
Acid from glucose	+	–	–	–	–
Acid from fructose	+	–	–	+	–
Biotin requirement	+	–	–	+	–
Nitrate reduction	+	+	d	+	d
Dentrification	d	d	–	+	–
Pectin hydrolyzed in 7 days	–	–	–	–	+
Utilization of					
L-Arabinose	+	d	+	d	+
Citrate	+	–	–	d	–
D-Fructose	+	+	+	+	d
D-Galactose	+	d	+	–	+
D-Glucose	+	d	+	–	+
Glycerol	+	+	–	+	–
D-Mannitol	+	–	–	+	–
D-Mannose	d	–	+	+	+
D-Ribose	+	–	+	+	d
D-Sorbitol	+	–	–	–	–
Sucrose	–	–	+	–	+

Acidification of peptone-based glucose broth	+	–	–	–	–
Acid from glucose or fructose broth anaerobically	d	–	–	–	–
G + C content	69–70	70–71	67	69–70	64–67
DNA:DNA relatedness (in %)	72–100	100	97–100	100	71–100
150 MDa plasmid	+	–	ND	ND	ND
$T_{m(e)}$ vs. 23S rRNA of *Azospirillum brasilense* ATCC 29145^T	80–82.5	80–82.5	73.5–75.3	74–74.8	ND
Similarity in 16S rRNA vs. *Azospirillum lipoferum*, expressed in %	100%	ND	ND	ND	87%

Note: Symbols: +, positive for all strains; –, negative for all strains; d, variable among strains; ND, not determined.

[a] Data from different papers (see References 15, 26, 33, 36, and 52).