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Polyphasic Taxonomy of Symbiotic Rhizobia from Wild Leguminous Plants Growing in Egypt

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ABSTRACT. About 20 strains of rhizobia from wild legumes were characterized based on numerical analysis of phenotypic characteristics, nodulating ability, fatty acid methyl esters (FAME) and SDS-PAGE profiles of whole cell proteins. FAME analysis revealed that palmitic (16:0), stearic (18:0) and arachidonic (20:0) were detected in most of wild-legume rhizobia, the latter being uncommon in fatty acid profiles of *Rhizobium* and *Sinorhizobium*. Numerical analysis of FAME classified strains of wild-legume rhizobia into 9 clusters and one heterogeneous group. There was both agreement and disagreement with the clustering data based on phenotypic analysis and FAME analysis. Four strains were grouped together in the same cluster based on both methods. However, 4 another strains, which were placed in one cluster of phenotypic analysis, were distributed in several clusters after FAME analysis. SDS-PAGE of whole-cell proteins revealed that the rhizobial strains exhibited protein profiles with peptide bands ranging from 5–19 band per profile and showed molar mass of 110–183 kDa. As in the case of FAME analysis, numerical analysis of protein bands was compared with clustering of phenotypic analysis. Agreement of the two methods was obvious when clustering some strains but conflicted in the classification of some other strains. However, integration of the three methods could be the basis of a polyphasic taxonomy. The twenty strains of wild-legume rhizobia were finally classified as follows: 12 strains related to *Rhizobium leguminosarum*, 5 strains related to *Sinorhizobium meliloti* and 3 strains to *Rhizobium* spp. Rhizobia nodulating wild herb legumes are among indigenous strains nodulating crop legumes in cultivated as well as noncultivated lands.

Rhizobia are a group of bacteria that are genetically diverse and physiologically heterogeneous (Rice *et al*. 1994; Parker 2002). Despite the fact that there are almost 20 000 legume species, fewer than 30 species of rhizobia have been described so far, most of which are capable of nodule formation (Doyle 1994; Sprent 1995, 2001; Tighe *et al.* 2000). Symbionts of less than 10 % of the 750 legume genera being fully characterized, it is likely that further exploration of the rhizobial diversity may reveal the rhizobial nature of additional members of the β-proteobacteria and possibly other taxonomic classes (Moulin *et al.* 2002). Characterization of native rhizobia, isolated from indigenous (naturally growing) legumes, has important consequences for ecological investigations (Mahler and Wollum 1982; Ahmad *et al*. 1984; Zahran 1999). Effective rhizobia isolated from these legumes may be used to inoculate certain crop legumes (Wange 1989; Zahran 1998, 2001). To propose further changes in the taxonomy of this group of organisms, it is essential to do it on the basis of both phenotypic and genotypic traits with a relatively large number of strains to construct a polyphasic taxonomy (Graham *et al*. 1991; Vandamme *et al*. 1996; Diouf *et al*. 2000). Such a basis would thus provide a more realistic picture of the proper taxonomic situation of rhizobia. One problem of rhizobial taxonomy is the contradiction between different methods of analysis. For example, the clustering analysis of rhizobia isolated from tree legumes (Zhang *et al*. 1991), based on numerical taxonomy of phenotypic characteristics, was not in complete agreement with that based on polyphasic characterization, *e.g.*, lipopolysaccharide analysis (Lindström and Zahran 1993) and fatty acid methyl ester (FAME) profiles, plasmid profiles and DNA–DNA hybridization (Zahran 1997). Until recently, the taxonomy of rhizobia was carried out mainly on species isolated from a few temperate crop species. With more isolates from other regions and from a greater number of leguminous species examined, and with improved techniques, extra diversity among the legume-nodulating bacteria has been found (Moreira *et al*. 1998).

Fatty acids have been used extensively as taxonomic criteria for bacterial classification. The variability in chain length, double bond position, and substituent groups, proved to be very useful taxonomic markers (Suzuki *et al*. 1993). Cellular FAME content is a stable parameter when highly standardized cultural conditions are used. Whole-cell fatty acid analysis was regarded as part of the polyphasic description and identification of a new species (Jarvis and Tighe 1994; Jarvis *et al*. 1996; Vandamme *et al*. 1996). Several

workers (*e.g.*, Kuykendall *et al.* 1988; Yokota 1989; Zahran 1997) used FAME analysis as a taxonomic marker for rhizobia. Tighe *et al.* (2000) used the fatty acid profiles to evaluate the current classification status of 600 strains belonging to the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium* and *Agrobacterium*.

SDS-PAGE is one of the most widely used techniques to study protein-profile characteristics of rhizobial strains. Protein electrophoresis is a reliable method for characterization of numerous bacterial strains, therefore, it was used in bacterial identification and classification (Ohya *et al.* 1988; Vauterin *et al.* 1993). Whole-cell protein analysis, using gel electrophoresis, is a useful tool for characterization of rhizobia (Fuquary *et al*. 1984; Zahran 1992, 1997; Zahran *et al*. 1994). Costas (1992) obtained a correlation between the high similarity in whole-cell protein content and DNA–DNA hybridization.

About 70 isolates of root-nodule bacteria from wild legumes have been examined in our laboratory for their phenotypic characteristics and also for their ability to nodulate some cultivated legumes, *e.g.*, *Vicia faba*, *Pisum sativum*, *Vigna sinensis* and *Medicago sativa* (Zahran *et al.* 2000). In this study the nodulating strains of these wild-legume rhizobia (20 strains) were examined further for molecular characteristics (*e.g.*, fatty acid profiles and whole cell protein pattern analysis) as an attempt to construct a polyphasic taxonomy.

MATERIALS AND METHODS

Fatty acid analysis. The cellular fatty acids of 33 strains, among them 10 references (Table I), 3 strains from clover and 20 strains of wild-legume rhizobia, which nodulated the experimental crop legumes (Zahran *et al*. 2000), were examined. The bacteria were grown for 2 to 3 d on yeast-extract–mannitol (YMA) medium; fatty acids were extracted and determined according to Kuykendall *et al*. (1988). GLC was used for estimating the percentage of each fatty acid with a 5 % phenylmethyl silicon capillary column (0.32 × 0.25 mm). Peaks were automatically integrated, and percentage of individual fatty acids was calculated. The carrier gas flow rate was 5 mL/min, column temperature 175–240 °C, detector temperature 270 °C. The data of cellular fatty acids were numerically analyzed to constitute clusters for the given strains. The characters were coded 1 or 0 on the basis of presence or absence of the fatty acid for each strain, respectively. The final matrix contained 33 strains and 22 fatty acids. Cluster analysis was carried out by using Unweighed Pairwise Group using Mathematical Average (UPGMA). The method was done using statistical Package for Social Science (SPSS) software version 8.

Table I. *Rhizobium* reference strains

No.	Strain[a]	Host
R1	*Rhizobium leguminosarum* bv. *viceae* ICARDA441	*Vicia faba*
R2	*Sinorhizobium meliloti* ARC2	*Medicago sativa*
R3	*Rhizobium leguminosarum* bv. *phaseoli* ARC310	*Phaseolus vulgaris*
R4	*Rhizobium* sp. ARC400	*Lupinus termis*
R5	*Mesorhizobium ciceri* ARC200	*Cicer arietinum*
R6	*Rhizobium* sp. ARC617	*Arachis hypogaea*
R7	*Rhizobium leguminosarum* bv. *viceae* ARC203P	*Pisum sativum*
R8	*Rhizobium leguminosarum* bv. *trifolii* ARC101	*Trifolium alexandrinum*
R9	*Rhizobium* sp. ARC808	*Vigna sinensis*
R10	*Rhizobium* sp. ARCTL2	*Acacia saligna*

[a]Sources: ARC – *Agricultural Research Center* (Giza, Egypt); ICARDA – *International Center for Agricultural Research in the Dry Areas* (Aleppo, Syria).

Protein analysis. Strains were grown on TY broth with the composition (g/L): tryptone 5, yeast extract 3, $CaCl_2 \cdot 6H_2O$ 0.75 for 2 d at 30 °C. Proteins were analyzed according to Lipsanen and Lindström (1989), and Zahran (1992). Cells were precipitated by centrifugation at 133 Hz in 0.5 mol/L Tris-HCl buffer (pH 6.8), and diluted with 0.5 mL distilled water. Fifty-µL volumes were diluted with 150 µL sample buffer (0.5 mol/L Tris-HCl, pH 6.8; 2 % SDS; 5 % 2-mercaptoethanol, 10 % glycerol, 5 ppm bromophenol blue) and heated in boiling water for 5 min. Cell debris was removed by centrifugation at 200 Hz. Cell lysates were analyzed by electrophoresis in 1.5 gels of 12 % acrylamide, in *BioRad* Mini-protein II cell. The electrophoretic analysis was done in a running buffer (in %: glycine 1.5, Tris-base 0.3, SDS 0.1) using 20 µL cell lysate in each lane. The gels were then stained by Coomassie Brilliant Blue (in %: Coomassie Blue 0.7,

acetic acid 9.2, 2-propanol 45.4) overnight and destained for 3–5 h in destaining solution (7.5 % acetic acid, 5.5 % 2-propanol) with gentle shaking. The destaining solution was changed several times until the background color was removed. The molar mass of proteins was compared with markers (94, 67, 43, 20 and 14 kDa). Gels obtained after protein electrophoresis was analyzed using Gel-Doc 2000 *BioRad* instrument with quantity software package supplied by the manufacturer. All bands of proteins from each strain were arranged in descending order according to their relative front value (R_F) and coded 1 if the band was present and 0 if not. The final matrix contained 31 strains and 95 value. Cluster analysis was carried out as described in fatty acid analysis but using package software version 10.

RESULTS AND DISCUSSION

Fatty acid analysis. In addition to the phenotypic characters of wild-legume rhizobia (Zahran *et al.* 2000), the percentage of fatty acids of rhizobial isolates, which nodulated target leguminous crops, were determined (Table II). The fatty acid composition varied not only among isolates of different leguminous species but also within that of the same species. Rhizobia isolates and reference strains contained most of the tested fatty acids. The fatty acids 9:0, 13:1, 20:1, 20:2, and 22:0 were synthesized by a few isolates. The fatty acid 12:0 was detected in all of the examined isolates. Margaric acid (17:0) was not detected in any of the tested isolates. The computer analysis of fatty acids assisted in classifying our rhizobia strains (33 strains) into 9 clusters at 46 % similarity, in addition to a heterogeneous group. A dendrogram of the linkage among clusters shows that cluster 1 is the largest, consisting of 8 strains (Fig. 1; *cf.* Table II), two reference strains (R3 and R7), two strains from *Alhagi murarum* (AMB5 and AMB7), and one strain from each of *Trifolium alexandrinum* (TAP1), *Melilotus indicus* (MIB4), *Acacia nilotica* (ANN3) and *Sesbania sesban* (SNN11). All strains synthesized caproic acid (6:0), undecylic acid (11:0), lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0), oleic acid (18:1) and linolenic acid 18:3 (Table II). None of the strains in this cluster synthesized acids 9:0 and 13:1. Only the reference strain R3 synthesized behenic acid (22:0).

Cluster 2 contained two strains from *M. indicus* (MIN4 and MIN10). Both produced 14 of the 22 identified fatty acids, which were the same acids produced by all strains in cluster 1. The following acids were also synthesized: caprylic acid (8:0), myristoleic acid (14:1), pentadecylic acid (15:0), 15:1, palmitoleic acid (16:1), stearic acid (18:0) and arachidonic acid (20:0). Strains TRP2 from *Trifolium resupinatum*, AMB4 from *A. murarum* and TRB3 from *T. resupinatum*, together with the reference strain ARC101 (R8) from *T. alexandrinum* were not related to a definite cluster. They were intermediate between clusters 1–3. The fatty acids 11:0, 12:0, 16:0 and 18:1 were well represented in all strains of this group (Table II, Fig. 1). Strain TRB3 from *T. resupinatum* was the only strain that produced the unsaturated fatty acid 13:1 (Table II).

Cluster 3 included one strain from *M. indicus* (MIB1) and another one from *T. alexandrinum* (TAB6). The two strains produced 10 of the 22 identified fatty acids (6:0, 11:0, 12:0, 14:0, 14:1, 15:0, 15:1, 16:0, 18:2 and 20:0), in addition, caprylic acid (8:0) and the unsaturated acid 13:1 were produced by strains TAB6 and MIB1, respectively (Table II). It was observed that clusters 1–4, along with the heterogeneous group were linked together, constructing a major large group harboring 4 reference strains (R3, R5, R7, R8), 2 strains from *T. alexandrinum* (TAP1 and TAB6), and 11 strains of the 5 wild legumes studied (Tables II and IV, Fig. 1).

Two reference strains were included in cluster 4, namely ARCT2 from *Acacia saligna* (R10) and TAN2 from *T. alexandrinum*. Fourteen fatty acids were determined in all strains of this cluster (6:0, 11:0, 12:0, 13:0, 14:0, 14:1, 15:0, 16:0, 18:0, 18:1, 18:2, 18:3, 20:0 and 20:2). In addition, the reference strain (R10) produced the acid 8:0.

Cluster 5 included two strains, the reference strain ARC2 from *Medicago sativa* (R2) and ANN6 from *A. nilotica*. They formed 11 of the 22 determined fatty acids. The acids in this cluster were similar to those formed by cluster 4 strains, except for acids 14:1, 15:0 and 22:2. Further strain ANN6 from *A. nilotica* produced fatty acids 10:0 (capric acid), 14:1 and 16:1 (Table II).

The two strains comprising cluster 6 came from each of *A. murarum* (AMB1) and *A. nilotica* (ANN5). Each of them was able to synthesize most of the identified fatty acids (15 of 22). Linolenic acid (18:3) was detected in strain ANN5 (Table II, Fig. 1).

Cluster 7 included two strains, one from *M. indicus* (MIN5) and another from *A. nilotica* (ANN4). The saturated fatty acids 6:0, 11:0, 12:0 and 20:0 were produced by both strains contained in this cluster.

Strain TRP3 of *T. resupinatum* exhibited a fatty acid profile that resembles profiles of strains in cluster 7 and, accordingly, it is linked with this cluster at 46 % similarity (Fig. 1). Strain SSN7 from *Sesbania sesban* and the reference strain ARC808 from *Vigna sinensis* (R9), however, exhibited a fatty acid

Table II. Fatty acid percentage in rhizobial isolates and reference strains

Strain	Cluster[a]	Fatty acid																				
		6:0	8:0	9:0	10:0	11:0	12:0	13:0	13:1	14:0	14:1	15:0	15:1	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
R1	9	0	0	0	0	1.1	3.4	0	0	5.1	0	0	0	0	0	1.0	1.3	3.3	6.4	6.6	0	0	0
R2	5	1.5	0	0	0	2.2	8.2	1.2	0	2.7	0	0	0	5.0	0	7.3	6.6	10.8	15.0	19	0	4.8	3.4
R3	1	1.7	6.9	0	1.7	5.6	29.8	5.8	0	6.8	3.1	4.3	3.6	5.0	1.7	1.4	1.4	0.8	1.3	1.3	0	0	4.8
R4	4–9[b]	1.8	0	0	0	5.5	10.2	0	0	51.3	8.4	2.5	0	2.7	1.4	1.1	1.7	1.2	0	4.27	0	0	0
R5	1–2[b]	0	6.3	0	1.8	3.8	32.9	2.1	0	7.9	3.6	4.3	4.1	2	3.3	1.2	1.9	1.5	0	3.6	0	0	0
R6	9	0	0	0	0	2.3	5.8	3.2	0	3.6	2.4	0	0	8.3	0	3.5	2.2	5.2	2.4	49.3	0	0	0
R7	1	1.6	7.3	0	1.8	5.1	32.3	2.6	0	7.0	4.9	6.3	6	3.4	3.1	1.5	1.3	0	0.7	2.0	0	0	0
R8	H	1.8	0	0	0	6.2	12.1	2.6	0	47.1	6.2	3.2	1.4	10	0	4	2.9	0	0	0	0	0	0
R9	4–7[b]	3.3	0	0	2.7	0	2.3	0	0	2.3	0	0	0	9.7	0	14.1	2	19	5	23	2.4	13	0
R10	4	4	1.9	0	0	1.5	3.7	1.6	0	1	1.5	1.8	0	5	0	8.3	9	11	3.4	34.6	0	9.7	0
TAN2	4	1.1	0	0	0	2.1	8.6	1.6	0	1.3	1.3	3.2	0	5.4	0	9.2	1.6	19	2.5	21	0	10	0
TAB6	3	4.4	7	0	0	5.6	33.5	0	0	10	8.4	8	4.3	3	0	0	0	3	0	6	0	0	0
TAP1	1	2.2	6.6	0	2.2	7	30.1	2	0	7.6	5.2	6.8	5	2.5	3	1.5	2	0	2.4	3.5	0	0	0
TRN1	8	4.1	0	0	0	0	4.7	0	0	0	0	0	0	0	0	33	25.5	16.1	16.1	13.2	0	0	0
TRB3	H	0	4.3	4.3	1.7	4.2	26.4	6.2	2.2	10	6.7	7.4	4.6	2.2	4.1	0	1.6	0	0	5.2	0	0	0
TRP2	H	2.5	6.7	0	2.1	5.1	39	2.3	0	11.1	5.5	5.8	6.7	2.8	2.5	0	1.3	0	0	2	0	0	0
TRP3	U	4.1	0	0	0	0	5	0	0	2.3	0	0	0	6.6	0	10.8	2.4	22	0	33	0	11.7	11.7
MIN4	2	2.1	8.3	0	0	5.9	33.6	0	0	7.1	4.9	7.4	3.5	2.2	2.8	2	2.5	0	3.8	3.5	0	0	0
MIN5	7	10	0	0	0	3	17.4	0	0	0	0	0	0	4.6	0	8.2	1.2	13	0	33.3	0	8.2	8.2
MIN8	8	8.6	0	0	0	3.7	22	0	0	0	0	0	0	0	0	6	4.5	23	9	18.5	0	0	0
MIN10	2	4	6.4	0	0	5.5	26.3	0	0	8.3	2.6	7.4	3.6	2.5	3.7	3.1	3.1	2	4.5	7.1	0	0	0
MIB1	3	8.6	0	0	0	4	28	0	2.4	7.1	5.3	10.8	3.7	2.2	0	0	0	9.5	0	5.3	0	0	0
MIB4	1	2.4	8.2	0	1.7	4.6	33	2.1	0	6.4	3.4	3.7	3.4	2.6	1.6	1.7	2	1.5	4.1	4	6.9	0	0
AMB1	6	3.1	9.4	0	2.2	5.3	37.6	2	0	8.1	3.7	4.6	3.7	2.3	2	1.3	1.6	0	0	4.5	0	0	0
AMB4	H	6.4	0	0	0	1.7	8.6	0	0	0	0	0	0	5.5	7.4	2.1	12	6.3	18.7	0	8.1	0	0
AMB5	1	3.3	0	0	0	1.5	9	0	0	3.4	0	17	0	7.8	0	10.6	5.3	14.7	1.6	23.7	0	9.1	9.1
AMB7	1	2.3	0	0	0	0.8	5	0.8	0	3.1	0	0	0	7.7	12	1.3	19.6	1.4	28	0	12	2.5	2.5
SSN7	U	2.3	0	0	0	1	6	0.8	0	2.4	0	0	0	0	4.3	11.3	35	0	30	0	4.0	3.2	3.2
SSN11	1	1.5	8.2	0	2.5	6.6	33.1	2.2	0	7.5	5	4.8	4.1	3.3	2.2	0	2.4	0	2.6	3.3	0	0	0
ANN3	1	2.8	0	0	0	2	9.2	0.6	0	2.7	0	1.7	0	6.6	0	9.3	1.4	13.2	1.7	24.5	7	10.7	10.7
ANN4	7	2	7.9	0	0	4.4	26.9	3.15	0	10.1	9.6	8.7	2.3	0	2.9	3.1	0	0	0	2.3	0	0	0
ANN5	6	3.8	7.3	0	1.8	1.0	32	2.2	0	10.6	3.5	5.5	3.7	2.1	2.3	1.2	1.3	0	1.2	1.9	0	0	0
ANN6	5	2.8	0	0	8.4	2.3	6.7	11.6	0	2.2	2.2	5.4	2.3	1.1	3.6	2.2	2.1	4.9	3.6	8.15	0	0	0

[a]H – heterogeneous, U – unspecified. [b]Characteristics associated with indicated clusters.

composition that affiliates them to cluster 4 through 7. They formed the acids 6:0, 12:0 and 8:0 but neither the saturated acid 9:0 nor the acid 13:1. Thus both strains were linked to clusters 4 through 7 at 44 and 43 % similarity levels (Fig. 1).

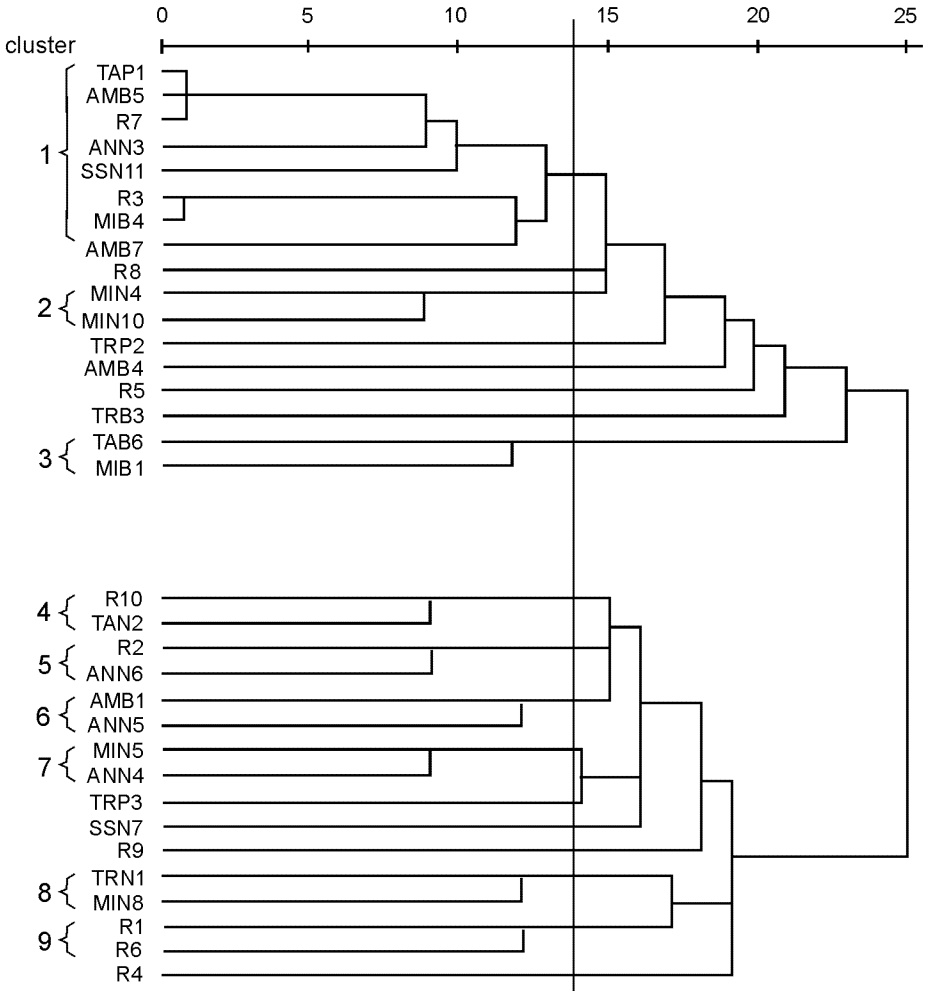

Fig. 1. Relationships among the nodulating rhizobial isolates from wild legumes and reference strains based on the average-linkage cluster analysis of fatty acid profile (22 fatty acids identified); *x*-axis – the average distances between the clusters; *vertical line* indicates the similarity level (46 %) at which the bacteria were classified into recognized clusters.

Cluster 8 contained two strains, one from *M. indicus* (MIN8), the other from *T. resupinatum* (TRN1). The general features of this cluster were the inability to produce most of the identified fatty acids. They produced only 6 acids (6:0, 18:0, 18:1, 18:2, 18:3, 20:0), but strain MIN8 synthesized the acid 11:0 (Table II).

Two references, ICARDA441 from *Vicia faba* (R1) and ARC617 from *Arachis hypogaea* (R6), were included in cluster 9 (Table II, Fig. 1). Eleven acids (11:0, 12:0, 13:0, 14:0, 14:1, 16:0, 18:0, 18:1, 18:2, 18:3, 20:0) were produced by both strains. The reference strain ARC400, from *Lupinus termis* (R4), produced the common fatty acids (*e.g.*, 12:0 and 18:0) but did not produce 13:1. These characteristics associated it with clusters 4–9 at 22 % similarity.

It is also noteworthy that the clusters 4–9 are linked together to form another major group in the dendrogram at 22 % similarity (Fig. 1). This group contained 6 reference strains (R1, R2, R4, R6, R9, R10), one strain (TAN2) from *T. alexandrinum* and 9 strains representing the five wild legumes dealt with in this study. Results of the fatty acid cluster analysis pointed out that the rhizobia of wild legumes are diverse, being classified into 9 different clusters. This conclusion is in agreement with the results of phenotypic cluster analysis (cf. Table IV; Zahran *et al.* 2000). Moreover, fatty acid analysis was done for only 33 strains of rhizobia, which may be inadequate to give a clear result.

Protein analysis. Protein profile analysis of the nodulating rhizobia strains from the wild legumes and the references were detected in SDS-PAGE. Computer analysis of protein bands and relative front values (Table III) of these profiles assisted to classify 31 rhizobial strains into four clusters at the similarity level of 38 %. A dendrogram showing the linkage between clusters was presented (Fig. 2). The peptide bands ranged from 5 to 19 bands and showed molar mass 9.9–189 of kDa corresponding to R_F = 0.01–1.

Table III. Protein band numbers, relative front distance (R_F) and molar mass (kDa) of different strains in 4 clusters

Strain	Cluster[a]	Strain code[b]	Band number	R_F	Molar mass
R2	1	1	9	0.20–0.97	11.2–94.0
R3	4	2	19	0.16–0.95	11.6–112
R4	1	3	10	0.48–0.98	10.9–36.9
R5	1	4	5	0.70–0.99	10.8–17.5
R6	1	5	7	0.39–0.90	12.0–54.5
R7	1	6	12	0.41–0.97	11.1–46.0
R8	1	7	10	0.17–0.69	11.3–108
R9	1	8	13	0.19–0.97	11.1–99.4
R10	H	9	17	0.11-0.95	11.5–140
TAN2	2	10	11	0.40–1	22.6–155
TAB6	2	11	12	0.11–1	10.4–121
TAP1	H	12	18	0.05–0.93	11.9–150
TRN1	U	13	12	0.05–0.99	10.9–146
TRB3	H	14	16	0.03–0.98	11.0–158
TRP2	U	15	13	0.07–0.98	11.1–139
TRP3	1	16	14	0.08–1	10.7–135
MIN4	H	17	16	0.08–0.98	11.1–132
MIN8	H	18	16	0.09–0.97	11.3–131
MIN10	1	19	10	0.23–0.97	10.4–78.8
MIB1	4	20	15	0.12–0.98	10.3–123
MIB4	U	21	17	0.20–0.91	9.9–88.0
AMB1	1	22	10	0.10–0.91	11.8–134
AMB4	1	23	13	0.10–0.98	10.4–135
AMB5	1	24	14	0.21–0.96	10.7–85.7
AMB7	1	25	11	0.02–0.98	10.4–185
SSN7	H	26	19	0.17–0.96	10.5–106
SSN11	3	27	13	0.01–1	9.9–189
ANN3	3	28	13	0.10–0.90	11.9–135
ANN4	H	29	16	0.14–0.91	11.8–156
ANN5	U	30	16	0.16–0.90	12.0–144
ANN6	1	31	10	0.41–0.90	11.9–52.0

[a]H – heterogeneous, U – unspecified. [b]Strain code number in the dendrogram (cf. Fig. 2).

Cluster 1 was the largest, it comprised 14 strains: 7 reference rhizobium strains (R2, R4, R5, R6, R7, R8, R9), one strain from each of *M. indicus* (MIN10), *A. nilotica* (ANN6) and *T. resupinatum* (TRP3), and 4 strains from *A. murarum* (AMB1, AMB4, AMB5, AMB7). The molar mass of the bands in this cluster ranged from 10.4 kDa (band number 13 in strain AMB4) to 183 kDa (band number 1 in strain AMB7) and corresponding to R_F values of 0.97 and 0.02, respectively.

Cluster 2 included two strains from *T. alexandrinum* (TAN2 and TAB6). Both strains have 11–12 peptide bands with molar mass ranging between 10.4 kDa (band number 12 in strain TAB6) to 155 kDa (band number 1 in strain TAN2), with R_F values corresponding to 0.1–1.0 and 0.4–1.0, respectively. Strain TRP2 (from *T. resupinatum*) was placed in cluster 1 through 2, while strain MIB4 from *M. indicus* was related to cluster 3 and both were joined together at 42 % similarity.

Cluster 3 contained two strains, from *S. sesban* (SSN11) and *A. nilotica* (ANN3). Both formed 13 peptide bands with molar mass 9.9–134 kDa. Strain TRN1 from *T. resupinatum* and ANN5 from *A. nilotica* was assigned together at 42 % similarity level and both were joined to clusters 1 through 3 at 42 % similarity.

The two strains of cluster 4 were the reference strain ARC310 from *Phaseolus vulgaris* (R3), which synthesized 19 peptide bands with molar mass 11.6–112 kDa, and MIB1 from *M. indicus*, which formed 15 peptide bands with molar mass 10.3–123 kDa.

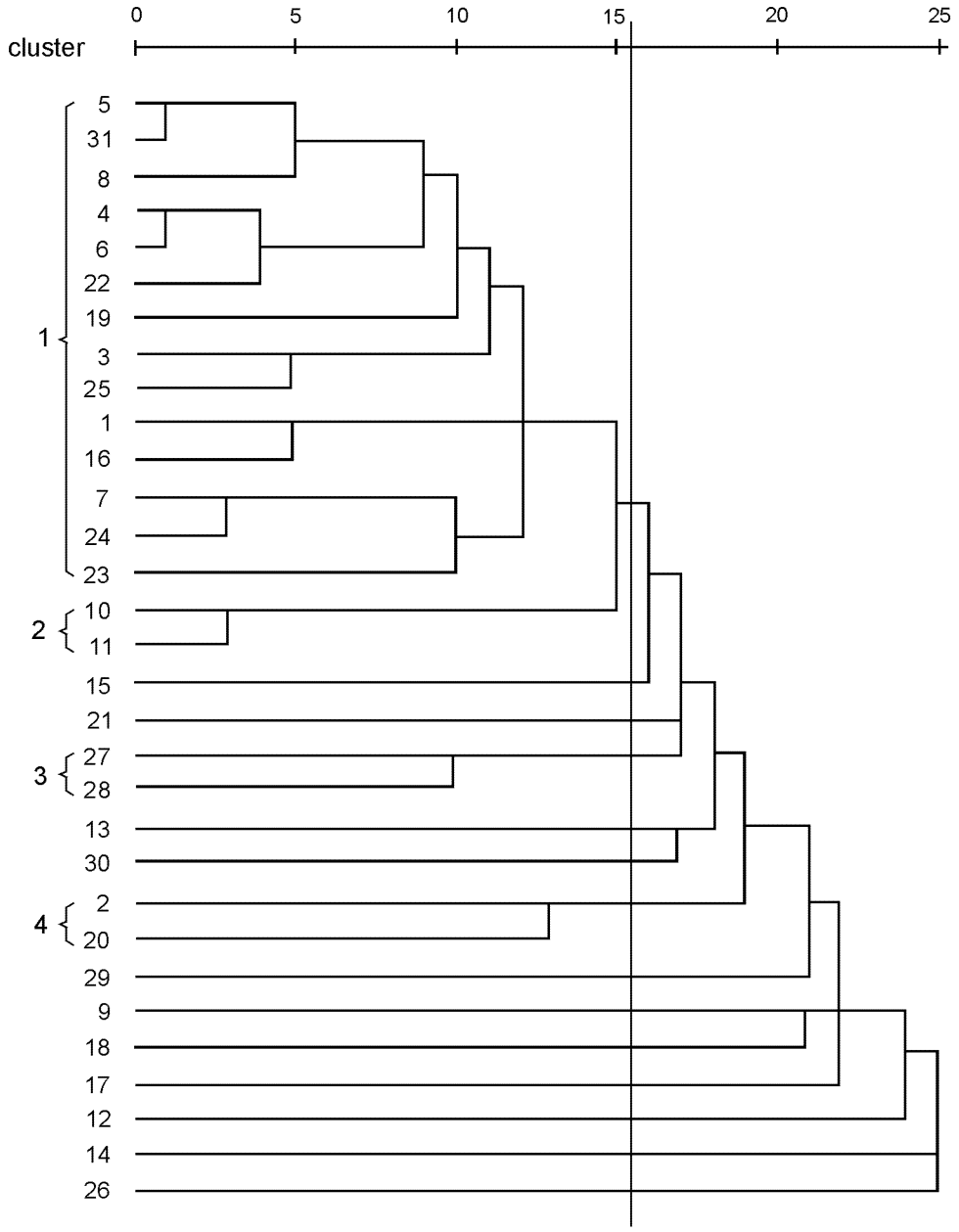

Fig. 2. Average-linkage cluster analysis of protein pattern relationships among the nodulating rhizobial isolates from wild legumes and reference strains; *x*-axis – the average distances between the clusters; *left* – strain code numbers (cf. Table III); *vertical line* indicates the similarity level (38 %) at which the bacteria were classified into recognized clusters.

The following strains, ANN4 from *A. nilotica*, the reference strain ARCTL2 from *Acacia saligna* (R10), strains MIN4 and MIN8 from *M. indicus*, strain TAP1 from *T. alexandrinum*, strain TRB3 of *T. resupinatum* and strain SSN7 from *S. sesban*, altogether comprised a heterogeneous group. These strains exhibited a protein pattern that is different from those obtained with other tested strains. They varied from 14 bands (in strain MIN8) to 19 bands (in strain SSN7). The molar mass of these bands lay between 10.6 kDa (band number 19 in strain SSN7) and 158 kDa (band number 1 in strain TRB3), corresponding to R_F values of 0.96 and 0.03, respectively. Analysis of protein profile pattern of our rhizobia strains led to the formation

of only 4 clusters; this is a low number of clusters compared to the 14 clusters of phenotypic characteristics (Zahran *et al*. 2000) and the 9 clusters of fatty acids (Table II, Fig. 1).

These results indicated that the rhizobia which were isolated from wild legumes (in this study) are less diverse with respect to their protein profile patterns. However, protein pattern analysis, can form, with other methods, a basis for polyphasic taxonomy (Tables IV and V).

Table IV. Clustering of rhizobial strains according to different analyses (phenotypic, fatty-acid and protein profile)[a]

Strain or isolate	Clustering based on analysis of			Strain or isolate	Clustering based on analysis of		
	phenotype[b]	fatty acids	proteins		phenotype[b]	fatty acids	proteins
TAN2	U	4	2	MIB4	1	1	U
TAB6	13	3	2	AMB1	7	6	1
TAP1	1	1	H	AMB4	7	H	1
TRN1	12	8	U	AMB5	7	1	1
TRB3	U	H	H	AMB7	7	1	1
TRP2	1	H	U	SSN7	9	U	H
TRP3	10	U	1	SSN11	8	1	3
MIN4	U	2	H	ANN3	1	1	3
MIN5	1	7	–[c]	ANN4	1	7	H
MIN8	1	8	H	ANN5	1	6	U
MIN10	1	2	1	ANN6	1	5	1
MIB1	4	3	4				

[a]H – heterogeneous, U – unspecified. [b]Zahran *et al.* (2000). [c]Not determined.

Analysis of cellular fatty acids has been regarded as a part of the polyphasic description and identification of new species (Jarvis and Tighe 1994; Jarvis *et al.* 1996; Vandamme *et al.* 1996). It has been reported that the FAME analysis is a very clear discrimination for genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium* and *Mesorhizobium* (MacKenzie *et al*. 1978; Kuykendall *et al.* 1988; So *et al.* 1994; Tighe *et al*. 2000). However, the results obtained from FAME analysis do not support the separation of *Sinorhizobium fredii* and *S. meliloti* into a separate genus (Jarvis *et al*. 1996). In the present study, rhizobia from wild legumes exhibited FAME profiles, which are generally similar to reference rhizobia (Table II, Fig. 1). The saturated fatty acids (palmitic 16:0 and stearic 18:0) were detected in the majority of strains of wild legumes and the references in relatively high amounts. These acids were reported to be dominant among the identified fatty acids of *R. trifolii* (Russa and Lorkiewicz 1974), in fast-growing rhizobia nodulating trees (Zahran 1997) and in some species of *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* (Tighe *et al*. 2000). In contrast to the previously reported in *Sinorhizobium* as well as in different species of *Rhizobium* (Tighe *et al*. 2000), the saturated fatty acid 17:0 was not detected in any of our strains. The majority of rhizobia from wild legumes and the reference strains synthesized the fatty acid arachidonic (20:0; Table II), which was not detected in any of the different species of *Rhizobium*, *Sinorhizobium* and *Mesorhizobium*, except for *M. ciceri*, *M. haukii*, *M. loti* and *M. pluriforium* studied by Tighe *et al*. (2000). It is essential, however, to use the same cultural and analytical conditions when comparing fatty acid profiles of different rhizobial strains (Kuykendall *et al*. 1988; Moore *et al.* 1994). FAME data (Table II, Fig. 1) were in agreement with other data (*e.g.*, phenotypic characteristics; Zahran *et al.* 2000) for the same rhizobial isolates. For example, the strains TAP1 from *T. alexandrinum* and ANN3 from *A. nilotica* were grouped together in cluster 1, based on fatty acid profiles and phenotypic characteristics (Fig. 1, Tables II and IV). Similarly, the strains MIN5 from *M. indicus* and ANN4 from *A. nilotica* with 15 common acids constructing cluster 7, were also grouped in cluster 1 with other rhizobial strains (*e.g.*, MIN8 and MIN10) of *M. indicus* in the phenotypically-based cluster analysis (Table IV). However, the two clustering methods were in conflict in certain cases. For example, the four strains of *A. murarum* (AMB1, AMB4, AMB5, AMB7), which were grouped in one cluster (cluster 7) by phenotypic analysis, exhibited a variety of fatty acid profiles and, as a result, showed different affiliations (AMB5 and AMB7 accommodated in cluster 1, AMB1 in cluster 6 and AMB4 in the heterogeneous group). On the other hand, rhizobia strains (MIN4 and MIN10) from *M. indicus* producing 14 common fatty acids of 22 acids (affiliated to cluster 2, were distributed in different clusters (MIN10 in cluster 1 and MIN4 was related to cluster 4 of the phenotypic analysis, Table IV).

Another comparison can be made for other strains (*e.g.*, TRN1 from *T. resupinatum* and MIN8 from *M. indicus*). These strains formed cluster 8, based on FAME analysis; nevertheless, they were classified in

different clusters, based on phenotypic characteristics (MIN8 classified in cluster 1 and TRN1 classified in cluster 12 of the phenotypic analysis). The disagreement between some of the results of fatty acid analysis and the phenotypically-based cluster analysis have previously been reported for the fast-growing tree rhizobia after chemotaxonomic study (Zahran 1997). However, analysis of cell fatty acids may result in more reliable data than those obtained by the traditional tests (Suzuki *et al*. 1993). Moreover, it is suggested that FAME profile analysis could be a useful tool for both determination of taxonomic positions of rhizobia and provide a good guide to phylogenetic relationships (Yokota 1989; So *et al*. 1994).

Table V. The suggested classification of different strains of wild rhizobia based on analysis of phenotypic relationships, fatty acid profiles, protein patterns and nodulation of legumes

Strain	Leguminous species	Suggested rhizobia species
TRN1	*Trifolium resupinatum*	*Rhizobium leguminosarum* bv. *trifolii*
TRB3		*ditto*
TRP2		*Sinorhizobium meliloti*
TRP3		*ditto*
MIN4	*Melilotus indicus*	*Rhizobium leguminosarum* bv. *phaseoli*
MIN5		*Sinorhizobium meliloti*
MIN8		*Rhizobium leguminosarum* bv. *viceae*
MIN10		*ditto*
MIB1		*Rhizobium leguminosarum* bv. *phaseoli*
MIB4		*ditto*
AMB1	*Alhagi murarum*	*ditto*
AMB4		*Rhizobium leguminosarum* bv. *trifolii*
AMB5		*Rhizobium leguminosarum* bv. *viceae*
AMB7		*Rhizobium* sp. (*Lupinus termis*)
SSN7[a]	*Sesbania sesban*	*Rhizobium* sp. (*Vigna sinensis*)
SSN11		*Rhizobium leguminosarum* bv. *viceae*
ANN3	*Acacia nilotica*	*Rhizobium leguminosarum* bv. *phaseoli*
ANN4		*Sinorhizobium meliloti*
ANN5		*ditto*
ANN6		*Rhizobium* sp. (*Arachis hypogaea*)

[a]Affiliated to *Rhizobium* sp. on the basis of phenotypic characterization and analysis of protein and fatty acid even though this strain failed to nodulate *Vigna sinensis* (Zahran *et al.* 2000).

Whole-cell protein analysis, using gel electrophoresis, has been reported to be a useful tool for characterization of rhizobia (Fuquary *et al.* 1984; Zahran 1992, 1997; Zahran *et al.* 1994). This technique has been used for the identification of slow-growing rhizobia of *Acacia albida* as members of the genus *Bradyrhizobium* (Dupuy *et al.* 1994). Moreover, SDS-PAGE of the whole-cell protein was used as a part of polyphasic characterization of rhizobia nodulating *P. vulgaris* (Diouf *et al.* 2000). *P. vulgaris* isolates were grouped into two main groups, I and II, which were related to *R. tropici* and *R. etli* (Diouf *et al.* 2000). In the present study, wild-legume rhizobia which were grouped in one cluster, based on protein pattern in SDS-PAGE similarity could be, but not always, members of the same cluster in numerical analysis, based on phenotypic relationships. Strains of *A. murarum* (AMB1, AMB4, AMB5, AMB7) were affiliated to the same cluster 1, based on similarity in protein profiles and also assembled together in cluster 7, based on phenotypic characteristics. Contradictory and conflicting data were also encountered. Thus, strains SSN11 from *S. sesban* and ANN3 from *A. nilotica* with 13 peptide bands and a range of molar mass 9.9–189 and 11.9–139 kDa, respectively, constructed cluster 3, based on protein profile analysis (Table III). These strains were grouped in totally different clusters, based on phenotypic analysis (ANN3 in cluster 1 and SSN1 in cluster 8). Similarly, strains TAN2 and TAB6 from *T. alexandrinum* were grouped together in cluster 2 according to protein profile analysis (Fig. 2, Table III), but were found in two different clusters (TAN2 unspecified and TAB6 in cluster 13), based on phenotypic analysis (Table IV). The results of protein banding analysis can be considered as reliable and precise.

Vauterin *et al*. (1993) reported that electrophoretic separation of whole-cell proteins is a sensitive and rapid method and provides information on the similarity of strains within the same species or subspecies. Strains ANN4 from *A. nilotica*, MIN8 from *M. indicus* and TAP1 from *T. alexandrinum*, which shared the heterogeneous group in protein analysis, were affiliated in cluster 1 after analysis of phenotypic relations (Table IV). Contradiction of data obtained by different methods has also been found in fast-growing rhizobia

nodulating some trees (Zahran 1997). Similarly, considerable heterogeneity in protein patterns and phenotypic features of fast growing rhizobia isolated from *Acacia* and *Sesbania* species in Senegal, was reported (De Lajudie *et al*. 1994).

In general, the wild-legume rhizobia, which were grouped in one cluster, based on FAME profile analysis and protein pattern analysis, were not always members of the same cluster when classified using numerical analysis of phenotypic relationships. There is some agreement in classification of some strains, *e.g.*, AMB5 and AMB7 of *A. murarum* and the reference strain (R7); they were grouped in the same cluster after analysis of fatty acid and protein profiles. However, ANN6 and the reference R2, which were grouped in cluster 1 based on protein analysis, were joined to different clusters, based on phenotypic analysis (ANN6 in cluster 1 and R2 in cluster 3; Zahran *et al.* 2000). A similar finding was obtained for strains SSN11 from *S. sesban* and ANN3 from *A. nilotica*; they were grouped together with other strains in clusters 1 and 3 based on both FAME and protein pattern analysis. Nevertheless, they were grouped in different clusters after analysis of phenotypic characteristics (ANN3 in cluster 1 and SSN11 in cluster 8, Table IV). This disagreement between the cluster analysis of phenotypic characteristics, on the one hand, and protein pattern and fatty acid profiles, on the other hand, not only confirmed the diversity of rhizobia from wild legumes, but also made the exact classification a very difficult task. The methods that were collectively used in this study could be a basic criterion for the classification and affiliation of new rhizobial isolates. Based on the overall similarities of cross-nodulation, analysis of phenotypic relations, fatty acid profiles and protein patterns (Tables IV and V), our strains can be related to *Rhizobium leguminosarum* (12 strains), *Sinorhizobium meliloti* (5 strains), *Rhizobium* sp. ARC400 (*Lupinus termis* 1 strain), *Rhizobium* sp. ARC808 (*Vigna sinensis* 1 strain) and *Rhizobium* sp. ARC617 (*Arachis hypogaea* 1 strain). Further studies, including genotypic characteristics, *e.g*., DNA–DNA relatedness, rRNA–DNA hybridization and 16S rRNA analysis, *etc*., are required to determine the exact taxonomic situation of rhizobia from wild legumes grown in arid climates.

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