Analysis of cellular fatty acids and phenotypic relationships of *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* species using the Sherlock Microbial Identification System

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Previous studies have demonstrated that cellular fatty acid analysis is a useful tool for identifying unknown strains of rhizobia and establishing taxonomic relationships between the species. In this study, the fatty acid profiles of over 600 strains belonging to the genera *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* were evaluated using the gas-chromatography-based Sherlock Microbial Identification System (MIS). Data collected with the MIS showed that the three phylogenetically defined biovars of the genus *Agrobacterium* formed discrete clusters, whilst species belonging to the genus *Mesorhizobium* formed three subclusters which were easily distinguished. These three subclusters contained *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum*, *Mesorhizobium tianshanense* fatty acid group I and *Mesorhizobium plurifarum*, and *Mesorhizobium huakuii* and *Mesorhizobium loti*. The genus *Sinorhizobium* was composed of an individual position for *Sinorhizobium meliloti* and a large cluster comprising *Sinorhizobium fredii*, *Sinorhizobium saheli*, *Sinorhizobium terangae*, *Sinorhizobium kostense* and *Sinorhizobium arboris*. *S. meliloti* contained significantly higher levels of the fatty acid 19:0 cyclo ω 8cis and clustered with *Rhizobium* sp. (*Hedysarum coronarium*). However, discrimination between the species of genera *Sinorhizobium* and *Rhizobium* was a function of the concentration of 16:0 3-OH. The genus *Rhizobium* contained a single cluster containing *Rhizobium* sp. (*Hedysarum coronarium*), *Rhizobium gallicum*, *Rhizobium leguminosarum* and *Rhizobium etli*, along with individual positions for *Rhizobium giardinii*, *Rhizobium tropici*, *Rhizobium galegae* and *Rhizobium hainanense*. *R. tropici* and *R. hainanense* exhibited similarity to *Agrobacterium* biovar 2, whilst *R. galegae* was similar to *Agrobacterium* biovar 1. *R. giardinii* appeared unique, with comparatively little similarity to the other species. Analysis of the genus *Bradyrhizobium* revealed large differences from the other genera studied. Two subgroups of *Bradyrhizobium elkanii* were detected and easily distinguished from *Bradyrhizobium japonicum*. *Bradyrhizobium liaoningense* and *Bradyrhizobium* sp. (*Arachis hypogaea*), a group isolated from Chinese peanut plants, showed similarities to *B. japonicum*, whilst a subgroup of *M. tianshanense* appeared identical to *Bradyrhizobium* sp. (*Arachis hypogaea*).

Keywords: *Rhizobium* taxonomy, fatty acid analysis, Sherlock Microbial Identification System

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Abbreviations: ECL, equivalent chain length; MIS, Microbial Identification System.

INTRODUCTION

Organisms collectively known as rhizobia are important plant-associated bacteria which have been studied extensively over the past twenty years. As the taxonomy of the rhizobia changes and additional species are described, the need for rapid comparisons and identification is necessary. In this study, we have characterized the cellular fatty acids of over 600 strains of rhizobia using the gas-chromatography-based Sherlock Microbial Identification System (MIS). This system is designed to analyse bacterial extracts for cellular fatty acids in an automated approach, and has been shown to be very sensitive and reproducible (Sasser, 1990). It was employed in this study because of the MIS software's library generation capability, the large number of known compounds in the system's peak naming table library, the ability to compare a large number of strains over a period of time, and the routine use of this system for bacterial analysis in many laboratories.

Phylogenetic data place the rhizobia and agrobacteria in the α -subdivision of the subclass *Proteobacteria*, and divide them into seven genera: *Rhizobium*, *Agrobacterium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, *Azorhizobium* and *Bradyrhizobium*. Members of the genera *Rhizobium* and *Sinorhizobium* are significant because of their ability to nodulate the roots of leguminous plants and carry out nitrogen fixation (Keyser *et al.*, 1982; Long, 1989; Lynch, 1976). Although this is also true of the genus *Bradyrhizobium*, their *in vitro* cellular proliferation rate is slower than that of the genus *Rhizobium* and they are genetically different from the other rhizobia (Jordan, 1982). Currently, there are four recognized species belonging to the genus *Rhizobium* which have been validated using polyphasic taxonomy; these are *Rhizobium etli*, *Rhizobium galegae*, *Rhizobium leguminosarum* and *Rhizobium tropici*. Additional studies have revealed that six additional species exist, these include *Rhizobium hainanense* (Chen *et al.*, 1997), *Rhizobium huautlense* (Wang *et al.*, 1998), *Rhizobium giardinii* (*Phaseolus* genomic species 2 type H152), *Rhizobium gallicum* (*Phaseolus* genomic species 1 type R602) (Amarger *et al.*, 1997), *Rhizobium mongolense* (Van Berkum *et al.*, 1998) and possibly *Rhizobium* sp. (*Hedysarum coronarium*) (Casella *et al.*, 1986) although this organism has not yet been validated.

Recent changes to the genus *Rhizobium* include the transfer of *Rhizobium loti* and *Rhizobium huakuii* to the new genus *Mesorhizobium*. The genus *Mesorhizobium* was proposed to accommodate the meso-growing rhizobia related to *R. loti*. The current species belonging to this genus include *Mesorhizobium loti*, *Mesorhizobium huakuii*, *Mesorhizobium mediterraneum*, *Mesorhizobium ciceri*, *Mesorhizobium tianshanense* (Jarvis *et al.*, 1997), *Mesorhizobium plurifarium* (de Lajudie *et al.*, 1998b) and *Mesorhizobium amorphae* (Wang *et al.*, 1999).

In 1988 the genus *Sinorhizobium* was proposed to

accommodate fast-growing nodule-forming soybean rhizobia (Chen *et al.*, 1988) and until recently contained only *Sinorhizobium fredii*, *Sinorhizobium xinjiangense* (Chen *et al.*, 1988), and *Sinorhizobium meliloti*. However, over the past several years, this genus has expanded to include *Sinorhizobium teranga* and *Sinorhizobium saheli* (de Lajudie *et al.*, 1994), *Sinorhizobium kostiense* and *Sinorhizobium arboris* (Nick *et al.*, 1999) and *Sinorhizobium medicae* (Rome *et al.*, 1996). Although *S. xinjiangense* was described some time ago, it is controversial due to the lack of polyphasic data and close similarity to *S. fredii* (Jarvis *et al.*, 1992). In this study, we did not evaluate strains of *S. medicae* or *S. xinjiangense*.

Unlike the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Allorhizobium* and *Azorhizobium*, members of the genus *Agrobacterium* are not capable of nodulating the roots of leguminous plants, but instead are considered plant pathogens capable of causing gall disease and root hair disease (Bradbury, 1986; Jordan, 1984). Species belonging to this genus were previously separated based on phytopathogenicity data and included *Agrobacterium tumefaciens*, *Agrobacterium radiobacter*, *Agrobacterium rhizogenes*, *Agrobacterium rubi* and *Agrobacterium vitis*. More recent work indicates that classification should be based on the three phylogenetically defined biovars rather than phytopathogenicity (Willems & Collins, 1993). This proposal has recognized biovar 1, 2 and 3 as *A. tumefaciens*, *A. rhizogenes* and *A. vitis*, respectively. Cellular fatty acid and phylogenetic data are in agreement and support the biovar classification (Jarvis *et al.*, 1996; Sawada *et al.*, 1992; Yokota *et al.*, 1993).

The taxonomy of the genus *Bradyrhizobium* has not experienced the rapid changes that have occurred in other genera. *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* have been established species for some time (Young & Haukka, 1996), but recent studies have proposed a new species, *Bradyrhizobium liaoningense* (Xu *et al.*, 1995). *B. liaoningense* was proposed for extra-slow-growing bradyrhizobia which exhibited unique phenotypic and genotypic characteristics. Studies performed on isolates recovered from the root nodules of peanuts (*Arachis hypogaea*) have also been investigated (Zhang *et al.*, 1999) and are presented in this manuscript for purposes of comparison with the other species. Although the genus *Bradyrhizobium* is not considered a close relative of other rhizobia, it was included in this study because (1) a large number of strains were analysed by methods identical to those used in the analysis of the other rhizobia, (2) several unique clusters were detected, and (3) results indicated that several strains of *M. tianshanense* appeared to belong to the genus *Bradyrhizobium*.

Also included among the rhizobia are the genera *Azorhizobium*, which contains the single species *Azorhizobium caulinodans*, and *Allorhizobium* which

contains the single species *Allorhizobium undicola* (de Lajudie *et al.*, 1998a). These organisms, however, were not evaluated in this study and data are not presented.

METHODS

Bacterial strains and growth conditions. All strains analysed in this study were obtained from established culture collections and had been previously characterized using phylogenetic techniques, metabolic characterization, protein gel electrophoresis, DNA–DNA hybridization and/or numerical analysis. All strains were grown using methods previously described (Graham *et al.*, 1995; Jarvis & Tighe, 1994).

All strains belonging to the genus *Agrobacterium* were received from the Culture Collection of the Laboratorium voor Microbiologie, Ghent, Belgium (LMG). *Agrobacterium* biovar 1 strains were LMG 187t, LMG 196, LMG 317, LMG 26, LMG 64, LMG 147, LMG 228, LMG 14, LMG 296T1t, LMG 176, LMG 340, LMG 305, LMG 31, LMG 139, LMG 140, LMG 200, LMG 226, LMG 27, LMG 201, LMG 225, LMG 296T2, LMG 146, LMG 198, LMG 15, LMG 181, LMG 290, LMG 267, LMG 232, LMG 62, LMG 148, LMG 338, LMG 177, LMG 186 and LMG 142. *Agrobacterium* biovar 2 strains were LMG 155, LMG 231, LMG 219, LMG 150, LMG 168, LMG 229, LMG 162, LMG 149, LMG 161, LMG 152, LMG 172, LMG 154, LMG 341, LMG 307, LMG 253 and LMG 63. *Agrobacterium* biovar 3 strains were LMG 257, LMG 8749, LMG 8750T, LMG 260, LMG 256, LMG 258, LMG 259, LMG 261, LMG 262 and LMG 8748.

All strains of *B. japonicum* and *B. elkanii* were received from the University of Minnesota (U of M) Soil Sciences Department. *B. elkanii* strains were UMR 40 (USDA 61), UMR 223 (IRC353C), UMR 130 (USDA 130), UMR 224 (IRC353B1), UMR 96 (S5), UMR 127 (BTIRC20), UMR 48 (USDA 31), UMR 128 (BT-2177A), NA6734, NA6742, NA6726, NA6733, CIAT 2335, NA6741, CK127, CK115, CK121, CK119, UMR 15 (USDA 40), NA6705, CK1, NA6710, NA6711, NA6708, CB3097, NA6728 and NA6809. *B. japonicum* strains were UMR 12 (USDA 126), NA6407, UMR 115 (USDA 115), UMR 90 (WB69), UMR 230 (USDA 141), UMR 23 (CB1809), UMR 25 (USDA 140), UMR 110 (USDA 110), UMR 6 (RCR3425), UMR 92 (CB2966), UMR 88 (CB2811), UMR 39 (USDA 125), UMR 62 (USDA 38), UMR 16 (USDA 62), UMR 21 (USDA 44), UMR 123 (USDA 123), NA6471, USDA 129, TAL1812, CK215, TAL1905, TAL1843, NA6721, NA6460, CK143, CK147, 1188, 1189, CB3053, CB283, WU253, CIAT 3144, USDA 454, USDA 467, CIAT 4103, CIAT 4967, CIAT 4100, CIAT 4099, CIAT 861, USDA 450, USDA 469, CIAT 3649, CIAT 3918 and CIAT 2434. The strains of *B. liaoningense* received from the Soil and Fertilizers Institute (SFI), CAAS, Beijing, Peoples' Republic of China, were 2043, 2064, 2061, 2068, 2281, DE544, 2062 and 2309. Strains of *Bradyrhizobium* sp. (*Arachis hypogaea*) were received from Sichuan Agricultural University, Peoples' Republic of China and the University of Helsinki, Department of Applied Chemistry and Microbiology, Helsinki, Finland (HAMBI). Strains were Spr 3-7, Spr 7-8, Spr 4-2, Spr 6-3, Spr 3-5, Spr 2-9, Spr 2-8, Spr 7-9, Spr 3-6, Spr 7-5, Spr 4-1, Spr 3-4, Spr 4-4, Spr 4-6, Spr 3-2, Spr 4-5, Spr 7-7, Spr 7-10, Spr 3-1, Spr 7-1, Spr 3-3 and Spr 4-10.

Strains of *M. ciceri* received from HAMBI were 522, IC 2091, UPM Ca116, 3H0a10, CP77, IC2018, UPM Ca7t (ATCC 51585), CP92 and CP76. Strains of *M. plurifarium*

received from the Institut de Recherche pour le Developpement (IRD) Dakar, Senegal, were ORS 1024, ORS 1026, ORS 1036, ORS 1032, ORS 1038, ORS 1015, ORS 1035, ORS 1030, ORS 1031, ORS 1029, ORS 1037, ORS 1010, ORS 1040, ORS 1001, ORS 654-204, ORS 656-205 and ORS 1018. Strains of *Mesorhizobium huakuii* received from the Culture Collection of Beijing Agricultural University (CCBAU), Peoples' Republic of China, were CBAU2609t, B23-106-26, 7034, T2, 38, 7034, B1940, 31, 9109, 9124, 7653, 9104, RA01, RA05, RA04 and SR98. Strains of *M. loti* were received from the American Type Culture Collection (ATCC) Manassas, VA, USA, and the Massey University Culture Collection (MUCC), Palmerston North, New Zealand, and were NZP2048, NZP2079, ATCC 33669, NZP2196 (SU343), NZP2048, NZP2227 (461), NZP2298 (L72M103c), NZP2037, NZP2230 (CC809a), NZP2235 (CC812a), NZP2146, NZP2234 (CC811), NZP2300, (L72M115c), NZP2241, NZP2203, NZP2195 and NZP2014. Strains of *M. mediterraneum* received from the Universite Claude Bernard-Lyon I (UCBL) Villeurbanne Cedex, France, were CP12, CP54, CP18, IC60, CP8, UPM Ca36t, CP14, Ca7, IC72M (ATCC 51586), CP28, H45 and CP24. Strains of *Mesorhizobium tianshanense* group I received from CCBAU were 005B, A-1-BS, 91x10, A6, 016Bm, 91x05, G2 and 91x11. Strains of *M. tianshanense* group II received from CCBAU were 91x13, 91x72, 91x07 and 91x09.

R. etli strains received from the Centro de Investigacion sobre Fijacion de Nitrogeno (CIFN), Cuernavaca, Morales, Mexico were F8, F17, F14, TAL182, 127K105, NITRAGIN 8251, VIK, CFN1, BRA5, 1G2, 2CH3, 2G18, 2G15, 2G11, MM13, 1CH9, 2M21, 1M11, 2G15, 2M20 and 1M18. Strains received from Institut National de la Recherche Agronomique (INRA) Dijon Cedex, France were BC45 (MSDJ 2291), BB13 (MSDJ 2249), BA12 (MSDJ 2223), CP115 (MSDJ 2531), GD112 (MSDJ 2398), BC15 (MSDJ 2277), CP134 (MSDJ 2535), CP91 (MSDJ 2488) and GD164 (MSDJ 2414). *R. galegae* strains received from HAMBI were 1460, 54013, 1428, 1145, 1146, 1143, 1141, 490, 1189, 1147, 1151, 1184, 1183, 1174, 1122, 1461, 1186, 503 and 1185. *R. gallicum* strains received from INRA were JSJ45, FI27, PHP222 (MSDJ 1100), R602 (MSDJ 1081), DC22 (MSDJ 114), PHD12 (MSDJ 960), JSJ18, JSJ42, JSJ55, JSJ15, 118H1, JSJ46, PHP18, Sm2, JSJ89, 116A15 and JSJ560. Strains of *R. giardinii* received from INRA were JSJ-Rt27, Ro67 (MSDJ 67), Ro84 (MSDJ 84), H236 (MSDJ 498), H231 (MSDJ 493), Ro70 (MSDJ 70), H251 (MSDJ 505), H152 (MSDJ 444), Ro17 (MSDJ 17), Ro7 (MSDJ 7), PHF28 (MSDJ 1084), Ro68 (MSDJ 68), Ro52 (MSDJ 52), H153 (MSDJ 447), Ro75 (MSDJ 75), Ro53 (MSDJ 53), Ro61 (MSDJ 61), H241 (MSDJ 499), H325 (MSDJ 707) and JSJ3. *R. hainanense* strains received from the CCBAU were I66, S25, I27, H14, I65, I33, I36, I20, I54, I2, I32, I64 and I12. *Rhizobium* sp. (*Hedysarum coronarium*) strains received from the Università Degli Studi di Padova (UDSP), Padova, Italy were RHF, IS123, RH44, HCNT1, RH19, A10, 835, S6, RH100, MS19, RHAL, VIL3-1, 30-21, VIL2, CC1337, VIL3-2, HA7, 47 and HCNA. *R. leguminosarum* strains received from MUCC were F310 (MU367), NZP1/6 (MU224), K8 (MU258), F300 (MU362), NZP5459 (MU273), M1928 (?), B248 (?), NZP5262 (CB596, MU272), NZP540 (SU39, MU269), SP18, NZP5230 (TA101, MU268), NZP5225 (MU267), TA1 (MU237), NZP514 (MU231), MU252, NZP5479 and MU632. Strains received from the ATCC were ATCC 14479 and ATCC 14480. Strains received from INRA were H242 (MSDJ 500), H136

(MSDJ 434), PhC152 (MSDJ 1083), Ro34 (MSDJ 34), PhF32 (MSDJ 1102), Ro29 (MSDJ 29), PhP17 (MSDJ 961), Ro35 (MSDJ 35) and H123 (MSDJ 429). *R. tropici* strains received from the CIFN were BR859, 10043, BR845, BR836, BR847, BR842, BR864, CFN299, BR863, BR10042, BR862, BR857, BR846, CIAT 899t, C-05-1, BR852, BR850 and BR853. Strains received from the INRA were CESTAS 221 (MSDJ 2932), CESTAS 114 (MSDJ 2870), CARA 56 (MSDJ 4573), AIREL 35 (MSDJ 4571), CESTAS 134 (MSDJ 2873), AIREL 27 (MSDJ 4569), AIREL 28 (MSDJ 4452) and BA45 (MSDJ 2241).

S. fredii strains received from MUCC were MU640 (USDA 217), MU646 (USDA 257), MU636 (USDA 191), MU643 (USDA 192), MU637 (USDA 194) and MU638 (USDA 298). Strains received from the CCBAU were CCBAU 116, CCBAU 110, CCBAU 112, CCBAU 105, CCBAU 103, CCBAU 115 and CCBAU 169. Strains received from the John Innes Institute, Norwich, UK were USDA 193, USDA 205t, USDA 201, USDA 206 and USDA 214. *S. meliloti* strains received from the MUCC were MU462 (U45), MU442 (CC2017), MU463 (300a13), MU461 (PDDCC1322), MU464 (Balsac), MU443 (CB1170), MU663 (2011) and MU441 (SU47). Strains received from Pennsylvania State University, PA, USA were M161, CC2003, 102FSI, 74B12, M275, 15A6, CC2013, M205, U102 and M1. Strains received from the ATCC were ATCC 10310 and ATCC 9930. *S. saheli* strains received from HAMBI were 1496 and 1495. Strains received from IRD were ORS 609t, ORS 611, ORS 600 and ORS 12. *S. teranga* strains received from IRD were ORS 1057, ORS 1071, ORS 1007, ORS 1013, ORS 1058, ORS 22, ORS 53, ORS 1009, ORS 604, ORS 51, ORS 52, ORS 1047, ORS 1025, ORS 1016, ORS 1073 and ORS 613. *S. kostiense* strains received from HAMBI were 1492, 1491, 1496, 1498, 1481, 1486, 1493, 1504, 1501, 1485, 1502, 1505, 1489, 1484, 1488, 1483, 1490, 1482 and 1679. *S. arboris* strains received from HAMBI were 1680, 1704, 1707, 1396, 1706, 1685, 1552, 1708 and 1700.

Analysis of cellular fatty acids. Harvesting and extraction procedures were performed according to methods described by Sasser (1990). Fatty acid analysis was performed using a Hewlett Packard 5890 series II gas chromatograph equipped with an Ultra2 capillary column. Construction of the microbial library and similarity data were accomplished using the MIS Sherlock software (Microbial ID). Three-dimensional plotting of principal components was performed using standard computer automated design software using coordinates from the MIS 2-D principal component plotting data.

The fatty acid compounds for each strain were identified by first converting the total cellular fatty acids to fatty acid methyl esters. Although some research has shown that the Sherlock MIS extraction method for converting cellular fatty acids to the fatty acid methyl ester derivatives may produce artifacts for rhizobia isolates (Orgambide *et al.*, 1993), the technique was performed throughout our research to preserve the standard operating procedure recommended by the Sherlock MIS suppliers. Identification of each fatty acid compound was determined using gas chromatography followed by data analysis with the Sherlock MIS software. Cellular fatty acids were identified by comparing the equivalent chain length (ECL) of each compound to a peak naming table that contains over 115 known standards. The quantity of each compound in a strain was determined as a percentage of the total amount of fatty acid compounds present for that strain.

RESULTS

The MIS library generation software was used to produce the mean fatty acid profiles in Tables 1–4. These tables indicate the mean percentage concentration of each compound detected in each species and were used by the MIS cluster analysis software to produce the coordinates in the principal component plot of Fig. 1. The cluster analysis software was further used to compute values presented in the similarity matrix using the identification values of all strains. Identification values are not included in this manuscript due to the large quantity of data they encompass.

In our previous work on the cellular fatty acids of rhizobia (Jarvis *et al.*, 1996; Jarvis & Tighe, 1994), several compounds were detected, but not successfully identified by the MIS software. Since that time, additional research has identified the compound 11 methyl 18:1 ω 7 cis , which appears to contribute to the discrimination of many species belonging to the genera *Rhizobium* and *Mesorhizobium*. Additionally, six new compounds were detected during this study from the new species examined. These compounds were 16:1 ω 5 cis , 18:1 ω 5 cis , unknown (ECL 14.780), unknown (ECL 15.953), unknown (ECL 17.875), and unknown (ECL 18.794). It is important to note that when evaluating large groups of previously unexamined strains, the detection of new compounds is not uncommon and it is important for them to be included in current work for establishing accurate relationships between groups.

Although some of the new compounds encountered in this study have not yet been identified, they have been programmed into our MIS database as unknown compounds with the corresponding ECL value. Compounds that eluted from the chromatography column at essentially the same retention time were grouped into one feature known as a 'summed feature' by the Sherlock MIS software. Although this situation could in theory have been circumvented by the use of a longer, more efficient capillary column, it was not possible in practice because the entire MIS fatty acid peak naming table and data analysis software has been standardized to the current capillary column. Confirmation of fatty acid compounds using mass spectrometry or other methods was not attempted during this study and compound identification was solely determined by the MIS.

Fatty acid composition

The fatty acid compositions of the species analysed are shown in Tables 1–4. These tables depict all fatty acids detected at a mean concentration of greater than 0.01 %. Compounds in the table that are indicated with an asterisk were not used to produce the similarity matrix or 3-D principal component plot because they did not occur in all strains of the particular species and are not likely to be significant. Although insignificant concentrations of some compounds were detected, they were included in this manuscript to provide a

Table 1. Mean fatty acid concentration of *Agrobacterium* and *Bradyrhizobium* species analysed in this study

Values are percentages of the total amount of fatty acid compounds present for that species. Numbers in parentheses indicate standard deviation.

Fatty acid compound	<i>Agrobacterium</i>			<i>Bradyrhizobium</i>				
	Biovar 1	Biovar 2	Biovar 3	<i>B. elkanii</i> group I	<i>B. elkanii</i> group II	<i>B. japonicum</i>	<i>B. liaoningense</i>	<i>Bradyrhizobium</i> sp. (<i>Arachis</i>)
10:0	ND	0.01 (0.05)*	ND	ND	ND	ND	ND	ND
10:0 3-OH	ND	0.24 (0.22)*	ND	ND	ND	ND	ND	ND
11:0 2-OH	ND	ND	ND	ND	ND	ND	ND	ND
Unknown (ECL 11-541)	ND	0.10 (0.11)*	ND	ND	ND	ND	ND	ND
Unknown (ECL 14-952)	ND	0.26 (0.16)*	0.13 (0.22)*	ND	ND	ND	ND	ND
12:0 3-OH	ND	ND	ND	0.02 (0.08)*	ND	0.04 (0.19)*	ND	ND
13:0 3-OH	ND	ND	ND	ND	ND	ND	ND	ND
13:1 at 12–13	0.49 (0.21)	0.37 (0.10)	ND	ND	ND	ND	ND	ND
13:0 iso 3-OH	ND	ND	ND	ND	ND	ND	ND	ND
14:0	0.11 (0.12)*	ND	ND	ND	ND	0.01 (0.07)*	ND	ND
Unknown (ECL 14-780)	0.06 (0.11)*	ND	0.22 (0.30)*	ND	ND	ND	ND	ND
15:0	0.29 (0.19)*	ND	ND	ND	ND	0.04 (0.15)*	0.13 (0.36)*	ND
15:0 anteiso	ND	ND	ND	ND	ND	ND	ND	ND
15:0 iso	ND	ND	ND	ND	ND	ND	ND	ND
15:0 2-OH	ND	ND	ND	ND	ND	ND	ND	ND
15:0 3-OH	0.03 (0.08)*	ND	ND	ND	ND	ND	ND	ND
15:0 iso 3-OH	ND	4.02 (0.54)	ND	ND	ND	ND	ND	ND
15:1 ω 8 cis	ND	ND	ND	ND	ND	ND	ND	ND
Unknown (ECL 15-953)	0.27 (0.15)*	0.28 (0.14)*	ND	ND	ND	ND	ND	ND
16:0	9.03 (1.29)	7.14 (1.98)	5.98 (0.91)	10.49 (2.35)	10.36 (0.60)	11.97 (1.70)	14.83 (1.48)	11.14 (1.79)
16:0 3-OH	4.76 (1.23)	5.19 (0.88)	2.28 (0.78)	ND	ND	ND	ND	ND
16:0 iso	ND	ND	ND	ND	ND	ND	ND	ND
16:1 ω 5 cis	ND	ND	ND	0.10 (0.27)*	ND	1.06 (0.99)	0.88 (0.18)	3.99 (1.69)
17:0	0.13 (0.14)*	0.06 (0.11)*	ND	0.03 (0.11)*	ND	0.19 (0.31)*	0.79 (1.06)	0.03 (0.13)*
17:0 iso	ND	0.60 (0.16)	ND	ND	ND	ND	ND	ND
17:0 iso 3-OH	ND	0.21 (0.18)*	ND	ND	ND	ND	ND	ND
17:0 cyclo	1.60 (0.54)	0.74 (0.36)	ND	0.95 (0.48)	0.50 (0.53)*	ND	ND	ND
17:0 3-OH	0.18 (0.18)*	ND	ND	ND	ND	ND	ND	ND
17:1 ω 6 cis	ND	ND	ND	ND	0.06 (0.22)*	0.15 (0.29)*	0.66 (0.88)	0.02 (0.09)*
17:1 ω 7 cis	ND	ND	ND	ND	ND	ND	ND	ND
17:1 ω 8 cis	0.22 (0.15)*	ND	ND	0.03 (0.09)*	0.05 (0.17)*	0.48 (0.52)	0.99 (1.10)	0.07 (0.17)*
Unknown (ECL 17-875)	ND	ND	ND	ND	ND	ND	ND	ND
18:0	0.17 (0.17)*	2.14 (0.57)	1.21 (0.48)	0.37 (0.29)	0.15 (0.26)*	0.62 (0.41)	0.38 (0.34)*	0.47 (0.50)*
11 methyl 18:1 ω 7 cis	0.23 (0.24)*	0.14 (0.17)*	0.39 (0.36)*	0.12 (0.31)*	ND	1.91 (3.16)	ND	ND
18:1 2-OH	ND	2.22 (0.87)	1.29 (0.43)	ND	ND	ND	ND	ND
18:1 ω 5 cis	ND	ND	ND	0.02 (0.08)*	ND	0.02 (0.09)*	ND	ND
18:1 ω 9 cis	ND	ND	ND	ND	ND	ND	ND	ND
Unknown (ECL 18-794)	0.01 (0.03)*	ND	ND	ND	ND	0.01 (0.08)*	0.27 (0.57)*	ND
19:0	ND	ND	ND	ND	ND	ND	ND	ND
19:0 cyclo ω 8 cis	18.78 (5.14)	34.62 (8.96)	4.11 (2.07)	19.99 (3.47)	8.82 (2.01)	1.16 (1.26)	5.04 (2.96)	0.19 (0.38)*
19:0 10 methyl	1.09 (0.30)	0.11 (0.18)*	2.03 (0.67)	ND	ND	ND	ND	ND
20:0	ND	ND	ND	ND	ND	ND	ND	ND
20:1 ω 9 $trans$	ND	0.04 (0.09)*	ND	ND	ND	ND	ND	ND
20:2 ω 6,9 cis	0.01 (0.04)*	0.13 (0.16)*	ND	0.29 (0.46)*	ND	ND	0.11 (0.32)*	ND
20:3 ω 6,9,12 cis	0.33 (0.16)	2.46 (0.50)	1.67 (0.38)	ND	ND	ND	ND	ND
Summed feature 2†	0.03 (0.08)*	ND	ND	ND	ND	ND	ND	ND
Summed feature 3‡	8.15 (1.90)	3.38 (0.44)	7.91 (2.57)	ND	ND	ND	ND	ND
Summed feature 4§	1.62 (0.40)	0.47 (0.19)	5.16 (1.16)	0.44 (0.38)	0.46 (0.42)	1.06 (0.68)	0.42 (0.45)*	0.44 (0.64)*
Summed feature 5	ND	ND	ND	0.10 (0.35)*	ND	ND	ND	ND
Summed feature 7¶	52.41 (6.86)	35.04 (10.78)	67.62 (4.85)	67.05 (5.04)	79.62 (2.55)	81.22 (4.66)	75.49 (3.97)	83.64 (2.04)

ND, Not detected.

*Compounds not used for calculating identification values, similarity indices or principal component values.

† Summed feature 2 = 15:1 iso H/I, 13:0 3-OH.

‡ Summed feature 3 = 12:0 (aldehyde?), unknown (ECL 10-928), 16:1 iso I/14:0 3-OH.

§ Summed feature 4 = 15:0 iso 2-OH, 16:1 ω 7 cis .

|| Summed feature 5 = 17:1 iso/anteiso B.

¶ Summed feature 7 = 18:1 ω 7 cis /18:1 ω 9 $trans$ /18:1 ω 12 $trans$, 18:1 ω 7 cis /18:1 ω 9 cis /18:1 ω 12 $trans$.

clearer representation of the fatty acids that could occur in each strain.

Agrobacterium. Fatty acid compounds detected in all biovars studied of the genus *Agrobacterium* included

16:0, 16:0 3-OH, 19:0 cyclo ω 8 cis , 20:3 ω 6,9,12 cis , summed feature 3, summed feature 4 and summed feature 7. Additional fatty acids detected in individual biovars included 13:1 at 12–13, 17:0 cyclo and 19:0 10 methyl in *Agrobacterium* biovar 1; 13:1 at 12–13, 15:0

Table 2. Mean fatty acid concentration of *Mesorhizobium* species analysed in this study

Values are percentages of the total amount of fatty acid compounds present for that species. Numbers in parentheses indicate standard deviation.

Fatty acid compound	<i>M. ciceri</i>	<i>M. huakuii</i>	<i>M. mediterraneum</i>	<i>M. loti</i>	<i>M. tianshanense</i> group I	<i>M. tianshanense</i> group II	<i>M. plurifarum</i>
10:0	ND	ND	ND	ND	ND	ND	ND
10:0 3-OH	ND	ND	ND	ND	ND	ND	ND
11:0 2-OH	ND	ND	ND	ND	ND	ND	ND
Unknown (ECL 11-541)	ND	ND	ND	ND	ND	ND	ND
Unknown (ECL 14-952)	ND	ND	ND	ND	ND	ND	ND
12:0 3-OH	0.27 (0.22)*	0.23 (0.31)*	0.11 (0.16)*	0.34 (0.36)*	0.09 (0.25)*	ND	0.20 (0.25)*
13:0 3-OH	ND	ND	ND	ND	ND	ND	ND
13:1 at 12-13	ND	ND	ND	ND	ND	ND	ND
13:0 iso 3-OH	0.24 (0.23)*	1.00 (0.26)	0.35 (0.28)*	1.23 (0.35)	0.27 (0.38)*	ND	0.84 (0.43)
14:0	ND	ND	ND	ND	ND	ND	ND
Unknown (ECL 14-780)	0.16 (0.25)*	ND	0.04 (0.15)*	ND	ND	ND	0.01 (0.06)*
15:0	0.02 (0.06)*	ND	0.04 (0.13)*	ND	ND	ND	ND
15:0 anteiso	ND	ND	ND	0.12 (0.51)*	ND	ND	ND
15:0 iso	0.18 (0.23)*	ND	0.13 (0.20)*	0.13 (0.25)*	0.16 (0.46)*	ND	ND
15:0 2-OH	ND	ND	ND	ND	ND	ND	ND
15:0 3-OH	ND	ND	ND	ND	ND	ND	ND
15:0 iso 3-OH	ND	ND	ND	ND	ND	ND	ND
15:1 ω 8cis	ND	ND	ND	ND	ND	ND	ND
Unknown (ECL 15-953)	ND	ND	ND	ND	ND	ND	ND
16:0	13.41 (2.11)	15.83 (0.98)	10.29 (1.39)	14.51 (2.15)	12.25 (2.26)	11.26 (1.11)	11.60 (1.53)
16:0 3-OH	ND	ND	ND	ND	ND	ND	ND
16:0 iso	ND	ND	ND	ND	ND	ND	ND
16:1 ω 5cis	ND	ND	ND	ND	ND	2.65 (0.53)	ND
17:0	1.29 (0.95)	0.42 (0.66)*	1.71 (1.77)	0.50 (0.59)	0.83 (1.02)	0.13 (0.26)*	0.69 (0.51)
17:0 iso	4.16 (0.69)	5.88 (1.13)	4.19 (1.14)	8.17 (1.93)	3.34 (0.68)	ND	6.26 (0.77)
17:0 iso 3-OH	ND	ND	ND	ND	ND	ND	ND
17:0 cyclo	0.84 (0.38)	0.70 (0.52)	0.85(0.82)	0.69 (0.51)	0.16 (0.31)*	ND	0.12 (0.23)*
17:0 3-OH	ND	ND	ND	ND	ND	ND	ND
17:1 ω 6cis	ND	ND	ND	ND	0.11 (0.32)*	0.26 (0.30)*	0.09 (0.26)*
17:1 ω 7cis	ND	ND	ND	ND	ND	ND	ND
17:1 ω 8cis	0.15 (0.23)*	0.25 (0.40)*	0.51 (0.65)*	0.12 (0.31)*	0.52 (0.77)*	0.14 (0.27)*	0.71 (0.50)
Unknown (ECL 17-875)	0.17 (0.52)*	ND	0.50 (0.99)*	ND	ND	ND	ND
18:0	3.25 (1.25)	4.10 (0.48)	4.21 (1.59)	4.50 (1.22)	4.99 (0.72)	0.92 (0.19)	2.99 (1.22)
11 methyl 18:1 ω 7cis	11.92 (3.61)	4.82 (2.77)	5.57 (4.33)	11.14 (2.48)	9.99 (1.38)	0.28 (0.55)*	ND
18:1 2-OH	ND	0.11 (0.32)*	ND	0.47 (0.62)*	ND	ND	ND
18:1 ω 5cis	ND	ND	0.06 (0.21)*	ND	ND	ND	ND
18:1 ω 9cis	0.12 (0.15)*	ND	ND	0.34 (0.42)*	ND	ND	ND
Unknown (ECL 18-794)	0.21 (0.38)*	ND	0.32 (0.49)*	0.08 (0.18)*	0.11 (0.31)*	ND	0.25 (0.35)*
19:0	ND	ND	ND	ND	ND	ND	ND
19:0 cyclo ω 8cis	37.27 (8.48)	22.96 (6.05)	33.35 (11.05)	21.93 (8.17)	12.37 (4.31)	0.57 (0.48)	11.33 (5.67)
19:0 10 methyl	0.18 (0.28)*	1.16 (0.45)	0.08 (0.19)*	1.23 (0.45)	0.16 (0.44)*	ND	1.28 (0.56)
20:0	0.05 (0.11)*	0.04 (0.15)*	ND	0.07 (0.19)*	ND	ND	0.04 (0.10)*
20:1 ω 9trans	0.14 (0.22)*	ND	0.46 (0.40)	0.15 (0.26)*	ND	ND	0.10 (0.16)*
20:2 ω 6,9cis	0.95 (0.68)	0.04 (0.15)*	0.77 (0.44)	0.05 (0.15)*	ND	ND	ND
20:3 ω 6,9,12cis	ND	ND	ND	ND	ND	ND	ND
Summed feature 2†	ND	ND	ND	ND	ND	ND	ND
Summed feature 3‡	ND	ND	ND	ND	ND	ND	ND
Summed feature 4§	0.34 (0.46)*	0.41 (0.50)*	0.61 (0.41)	0.51 (0.53)	1.42 (0.23)	0.29 (0.34)*	0.44 (0.45)
Summed feature 5	ND	ND	ND	ND	ND	ND	ND
Summed feature 7¶	24.69 (8.43)	42.04 (7.41)	35.81 (12.47)	33.73 (6.16)	53.22 (5.19)	83.52 (1.52)	63.02 (5.67)

For footnotes, see Table 1.

iso 3-OH, 17:0 iso, 17:0 cyclo, 18:0 and 18:0 2-OH in *Agrobacterium* biovar 2; and 18:0, 18:1 2-OH and 19:0 10 methyl in *Agrobacterium* biovar 3.

These data resulted in discrete clustering of strains belonging to *Agrobacterium* biovar 1, *Agrobacterium* biovar 2 and *Agrobacterium* biovar 3. Comparison of the three biovars indicated that *Agrobacterium* biovar 1 has a fatty acid profile similar to that of *Rhizobium* sp. (*Hedysarum coronarium*), but deviates by lacking 18:0 and by containing a higher concentration of 16:0 and 16:0 3-OH. *Agrobacterium* biovar 2 was similar to *R. hainanense* but differed by possessing a higher concentration of 15:0 iso 3-OH, 16:0 3-OH and

summed feature 4. *Agrobacterium* biovar 3 appeared to have a unique fatty acid composition with only a slight similarity to *R. giardinii*.

Mesorhizobium. Fatty acid compounds detected in all species of the genus *Mesorhizobium* included 16:0, 17:0, 17:0 iso, 18:0, 19:0 cyclo ω 8cis and summed feature 7. Additional fatty acids detected in individual species included 17:0 cyclo, 11 methyl 18:1 ω 7cis and 20:2 ω 6,9cis in *M. ciceri*; 13:0 iso 3-OH, 17:0 cyclo, 11 methyl 18:1 ω 7cis and 19:0 10 methyl in *M. huakuii*; 17:0 cyclo, 11 methyl 18:1 ω 7cis, 20:1 ω 9trans, 20:2 ω 6,9cis and summed feature 4 in *M. mediterraneum*; 13:0 iso 3-OH, 17:0 cyclo, 11 methyl

Table 3. Mean fatty acid concentration of *Rhizobium* species analysed in this study

Values are percentages of the total amount of fatty acid compounds present for that species. Numbers in parentheses indicate standard deviation.

Fatty acid compound	<i>R. etli</i>	<i>R. galegae</i>	<i>Rhizobium</i> sp. (<i>Hedysarum</i>)	<i>R. hainanense</i>	<i>R. leguminosarum</i>	<i>R. tropici</i>	<i>R. gallicum</i>	<i>R. giardinii</i>
10:0	ND	ND	ND	ND	ND	ND	ND	ND
10:0 3-OH	ND	ND	ND	0.08 (0.30)*	ND	ND	ND	ND
11:0 2-OH	0.02 (0.09)*	ND	ND	ND	ND	ND	ND	ND
Unknown (ECL 11-541)	ND	ND	ND	0.54 (0.10)	ND	0.02 (0.07)*	ND	ND
Unknown (ECL 14-952)	0.20 (0.30)*	ND	0.02 (0.07)*	1.09 (0.19)	ND	0.02 (0.08)*	0.25 (0.21)*	0.21 (0.45)*
12:0 3-OH	ND	ND	ND	ND	ND	ND	ND	ND
13:0 3-OH	ND	ND	ND	ND	ND	ND	ND	ND
13:1 at 12-13	ND	ND	0.16 (0.20)*	0.85 (0.16)	ND	0.07 (0.15)*	0.48 (0.28)	ND
13:0 iso 3-OH	ND	ND	ND	ND	ND	ND	ND	ND
14:0	ND	ND	ND	ND	ND	ND	ND	ND
Unknown (ECL 14-780)	ND	ND	ND	ND	ND	ND	ND	0.10 (0.32)*
15:0	ND	ND	ND	ND	ND	ND	ND	ND
15:0 anteiso	ND	ND	ND	ND	0.04 (0.21)*	ND	ND	ND
15:0 iso	ND	ND	ND	ND	0.03 (0.16)*	0.02 (0.09)*	ND	ND
15:0 2-OH	0.70 (0.57)	ND	0.09 (0.15)*	ND	0.87 (0.41)	ND	0.48 (0.16)	ND
15:0 3-OH	0.03 (0.11)*	ND	0.20 (0.22)*	ND	0.02 (0.09)*	ND	ND	ND
15:0 iso 3-OH	ND	ND	ND	2.94 (0.18)	ND	3.40 (0.46)	ND	ND
15:1 ω 8cis	ND	ND	ND	ND	ND	ND	ND	ND
Unknown (ECL 15-953)	ND	ND	0.01 (0.04)*	0.05 (0.11)*	ND	0.11 (0.15)*	ND	ND
16:0	2.53 (0.46)	9.72 (1.42)	4.60 (0.58)	6.33 (0.50)	4.08 (1.00)	7.70 (1.89)	3.22 (0.42)	11.39 (1.64)
16:0 3-OH	1.20 (0.22)	3.30 (0.29)	2.64 (0.20)	3.53 (0.31)	1.32 (0.28)	3.87 (0.24)	2.29 (0.17)	0.03 (0.12)*
16:0 iso	ND	ND	ND	ND	ND	ND	ND	ND
16:1 ω 5cis	ND	ND	ND	ND	ND	ND	ND	ND
17:0	0.32 (0.50)*	0.43 (0.41)	0.99 (0.51)	0.33 (0.36)*	0.32 (0.42)*	0.02 (0.12)*	0.09 (0.21)*	0.58 (0.47)
17:0 iso	ND	ND	ND	1.32 (0.33)	ND	0.93 (0.27)	ND	ND
17:0 iso 3-OH	ND	ND	ND	0.15 (0.20)*	ND	0.31 (0.22)*	ND	ND
17:0 cyclo	ND	0.11 (0.20)*	0.48 (0.25)	0.20 (0.23)*	0.15 (0.28)*	0.68 (0.42)	0.02 (0.09)*	1.77 (0.71)
17:0 3-OH	ND	0.09 (0.19)*	0.06 (0.11)*	ND	ND	ND	ND	ND
17:1 ω 6cis	ND	ND	ND	ND	ND	ND	ND	ND
17:1 ω 7cis	ND	ND	ND	ND	ND	ND	ND	ND
17:1 ω 8cis	0.03 (0.11)*	0.06 (0.15)*	0.24 (0.23)*	ND	0.01 (0.07)*	ND	ND	0.12 (0.25)*
Unknown (ECL 17-875)	ND	ND	ND	ND	ND	ND	ND	ND
18:0	8.71 (1.21)	1.20 (0.32)	5.75 (1.04)	3.98 (0.60)	7.83 (1.97)	2.87 (1.21)	9.26 (2.41)	2.35 (0.67)
11 methyl 18:1 ω 7cis	1.48 (2.02)	0.10 (0.19)*	0.02 (0.07)*	0.89 (0.37)	4.70 (2.65)	0.55 (0.24)	ND	2.87 (1.35)
18:1 2-OH	ND	ND	ND	1.95 (0.60)	0.05 (0.24)*	0.82 (0.66)	ND	ND
18:1 ω 5cis	ND	ND	ND	ND	ND	ND	ND	ND
18:1 ω 9cis	0.12 (0.30)*	ND	0.05 (0.08)*	ND	0.09 (0.21)*	ND	0.03 (0.06)*	ND
Unknown (ECL 18-794)	0.06 (0.19)*	0.02 (0.07)*	0.11 (0.15)*	ND	0.03 (0.11)*	ND	ND	ND
19:0	ND	ND	ND	ND	0.01 (0.06)*	ND	ND	ND
19:0 cyclo ω 8cis	10.19 (5.44)	31.12 (9.31)	20.00 (8.13)	38.84 (4.27)	13.91 (5.32)	49.29 (9.76)	7.30 (3.49)	2.78 (1.36)
19:0 10 methyl	0.01 (0.06)*	2.32 (0.36)	0.21 (0.17)*	ND	0.02 (0.12)*	ND	0.10 (0.16)*	1.53 (0.33)
20:0	ND	ND	ND	ND	ND	ND	ND	ND
20:1 ω 9trans	0.09 (0.16)*	ND	ND	ND	0.01 (0.05)*	ND	0.23 (0.18)*	ND
20:2 ω 6,9cis	ND	0.11 (0.17)*	0.05 (0.12)*	0.57 (0.13)	ND	0.39 (0.22)	ND	ND
20:3 ω 6,9,12cis	1.95 (0.37)	1.25 (0.19)	0.87 (0.06)	2.80 (0.42)	2.31 (0.53)	1.83 (0.65)	0.77 (0.22)	2.66 (0.49)
Summed feature 2†	ND	ND	ND	ND	ND	ND	ND	ND
Summed feature 3‡	5.95 (0.68)	4.82 (0.39)	6.42 (0.63)	3.30 (0.32)	6.46 (0.94)	2.33 (0.67)	6.25 (0.43)	10.45 (2.13)
Summed feature 4§	0.22 (0.24)*	0.57 (0.32)	1.06 (0.21)	0.09 (0.17)*	0.52 (0.35)	0.16 (0.23)*	0.58 (0.19)	4.37 (0.95)
Summed feature 5	ND	ND	ND	ND	ND	ND	ND	ND
Summed feature 7¶	66.17 (8.01)	44.78 (10.75)	55.99 (8.18)	30.16 (5.16)	57.22 (6.49)	24.61 (11.00)	68.65 (2.65)	58.80 (4.62)

For footnotes, see Table 1.

18:1 ω 7cis, 19:0 10 methyl and summed feature 4 in *M. loti*; 11 methyl 18:1 ω 7cis and summed feature 4 in *M. tianshanense* group I; and 13:0 iso 3-OH, 17:1 ω 8cis, 19:0 10 methyl and summed feature 4 in *M. plurifarum*. This genus could be distinguished from the other genera because it lacked 20:3 ω 6,9,12cis and summed feature 3 and possessed 17:0 iso fatty acids.

M. ciceri possessed a unique fatty acid profile with similarities to *M. mediterraneum*. It differed from other species belonging to this genus by containing a lower concentration of summed feature 7 and a higher concentration of 19:0 cyclo ω 8cis. This organism

separated from *M. mediterraneum* due to its higher concentration of the 11 methyl 18:1 ω 7cis. *M. mediterraneum* appeared to be similar to *M. ciceri*, but could be distinguished from the other species based on the concentration of summed feature 7 and 19:0 cyclo ω 8cis. Analysis of *M. tianshanense* strains revealed two groups. These groups were designated *M. tianshanense* fatty acid groups I and II. Group I appeared to belong to the genus *Mesorhizobium* with similarities to the '*M. ciceri*–*M. mediterraneum*' cluster. Unlike the other members of this genus, this group contained much less of 19:0 cyclo ω 8cis and a greater concentration of summed feature 7. Group II of *M. tianshanense*

Table 4. Mean fatty acid concentration of *Sinorhizobium* species analysed in this study

Values are percentages of the total amount of fatty acid compounds present for that species. Numbers in parentheses indicate standard deviation.

Fatty acid compound	<i>S. fredii</i>	<i>S. meliloti</i>	<i>S. saheli</i>	<i>S. terengae</i>	<i>S. kostiense</i>	<i>S. arboris</i>
10:0	ND	ND	ND	ND	ND	ND
10:0 3-OH	ND	ND	ND	ND	ND	ND
11:0 2-OH	ND	ND	ND	ND	ND	ND
Unknown (ECL 11-541)	ND	ND	ND	ND	ND	ND
Unknown (ECL 14-952)	ND	ND	0.26 (0.44)*	0.15 (0.25)*	0.37 (0.80)*	0.13 (0.39)*
12:0 3-OH	ND	ND	ND	ND	ND	ND
13:0 3-OH	ND	ND	ND	ND	ND	ND
13:1 at 12-13	ND	ND	ND	ND	ND	ND
13:0 iso 3-OH	ND	ND	ND	ND	ND	ND
14:0	ND	ND	ND	ND	ND	ND
Unknown (ECL 14-780)	ND	ND	ND	ND	0.06 (0.19)*	ND
15:0	ND	ND	ND	ND	ND	ND
15:0 anteiso	0.10 (0.44)*	ND	ND	ND	ND	ND
15:0 iso	0.01 (0.06)*	ND	ND	ND	ND	ND
15:0 2-OH	ND	ND	ND	ND	ND	ND
15:0 3-OH	ND	ND	ND	ND	ND	ND
15:0 iso 3-OH	ND	ND	ND	ND	ND	ND
15:1 ω 8cis	ND	ND	ND	ND	ND	ND
Unknown (ECL 15-953)	ND	ND	ND	ND	ND	ND
16:0	5.12 (1.34)	4.93 (1.14)	5.01 (1.34)	3.30 (0.93)	4.94 (1.17)	5.20 (1.08)
16:0 3-OH	0.49 (0.29)	0.68 (0.12)	0.61 (0.52)*	0.18 (0.24)*	0.03 (0.15)*	0.10 (0.29)*
16:0 iso	0.03 (0.13)*	ND	ND	ND	ND	ND
16:1 ω 5cis	ND	ND	ND	ND	ND	ND
17:0	0.73 (0.46)	0.54 (0.24)	0.08 (0.20)*	0.24 (0.29)*	0.56 (0.60)	ND
17:0 iso	ND	ND	ND	ND	ND	ND
17:0 iso 3-OH	ND	ND	ND	ND	ND	ND
17:0 cyclo	0.20 (0.35)*	0.75 (0.31)	0.15 (0.36)*	ND	0.04 (0.16)*	ND
17:0 3-OH	ND	0.05 (0.10)*	ND	0.02 (0.06)*	ND	ND
17:1 ω 6cis	ND	ND	ND	ND	ND	ND
17:1 ω 7cis	ND	ND	ND	ND	ND	ND
17:1 ω 8cis	0.11 (0.16)*	0.09 (0.14)*	ND	0.03 (0.08)*	ND	ND
Unknown (ECL 17-875)	ND	ND	ND	ND	ND	ND
18:0	4.99 (1.15)	2.69 (0.73)	1.08 (0.77)	3.44 (0.87)	4.02 (1.12)	3.91 (0.63)
11 methyl 18:1 ω 7cis	ND	0.02 (0.07)*	ND	ND	ND	ND
18:1 2-OH	ND	ND	ND	ND	ND	ND
18:1 ω 5cis	ND	ND	ND	ND	ND	ND
18:1 ω 9cis	0.11 (0.18)*	0.09 (0.07)*	ND	0.01 (0.03)*	0.05 (0.14)*	ND
Unknown (ECL 18-794)	0.19 (0.19)*	0.11 (0.16)*	ND	0.05 (0.13)*	ND	ND
19:0	ND	ND	ND	ND	ND	ND
19:0 cyclo ω 8cis	4.37 (3.63)	22.53 (8.24)	3.55 (2.25)	2.92 (2.00)	4.02 (2.18)	8.94 (4.99)
19:0 10 methyl	0.41 (0.32)	0.85 (0.26)	0.80 (0.65)	0.07 (0.16)*	0.67 (0.51)	ND
20:0	ND	ND	ND	ND	ND	ND
20:1 ω 9trans	0.12 (0.26)*	ND	ND	ND	ND	ND
20:2 ω 6,9cis	ND	0.02 (0.07)*	ND	ND	ND	ND
20:3 ω 6,9,12cis	3.28 (0.92)	2.94 (0.34)	3.74 (1.06)	2.00 (0.53)	2.98 (0.68)	3.43 (0.37)
Summed feature 2†	ND	ND	ND	0.01 (0.05)*	0.09 (0.22)*	ND
Summed feature 3‡	7.62 (2.26)	6.44 (0.70)	13.57 (3.31)	7.70 (2.28)	10.34 (1.87)	9.71 (2.73)
Summed feature 4§	0.63 (0.37)	0.34 (0.21)	1.24 (0.33)	0.42 (0.34)	0.50 (0.45)	0.14 (0.41)*
Summed feature 5	ND	ND	ND	0.08 (0.32)*	ND	ND
Summed feature 7¶	71.45 (5.25)	56.93 (8.25)	69.91 (4.50)	79.40 (3.27)	71.31 (4.31)	68.44 (8.16)

For footnotes, see Table 1.

appeared to belong to the genus *Bradyrhizobium* and possessed an almost identical fatty acid composition to *Bradyrhizobium* sp. (*Arachis hypogaea*). *M. plurifarum* was similar to *M. tianshanense* fatty acid group I, but contained a lower concentration of 17 iso and 18:0. It differed from other members of the genus because it contained the highest concentration of summed feature 7 and lacked 11 methyl 18:1 ω 7cis, which was detected in all other species of the genus *Mesorhizobium* at levels greater than 4%. The concentration of 19:0 cyclo ω 8cis was lower than the other species of the genus, but comparable to *M. tianshanense* fatty acid group I.

Rhizobium. Fatty acids detected in all species of the genus included 16:0, 18:0, 19:0 cyclo ω 8cis, 20:3 ω 6,9,12cis, summed feature 3 and summed feature 7. Additional fatty acids detected in individual species included unknown (ECL 11-541) and unknown (ECL 14-952), 13:1 at 12-13, 15:0 iso 3-OH, 16:0 3-OH, 17:0 iso, 11 methyl 18:1 ω 8cis, 18:0 2-OH and 20:2 ω 6,9cis in *R. hainanense*; 17:0, 17:0 cyclo, 11 methyl 18:1 ω 8cis, 19:0 10 methyl and summed feature 4 in *R. giardinii*; 13:1 at 12-13, 15:0 2-OH, 16:0 3-OH and summed feature 4 in *R. gallicum*; 15:0 iso 3-OH, 16:0 3-OH, 17:0 iso, 17:0 cyclo, 11 methyl 18:1 ω 8cis, 18:0 2-OH and 20:2 ω 6,9cis in *R. tropici*; 16:0 3-OH,

17:0, 19:0 10 methyl and summed feature 4 in *R. galegae*; 16:0 3-OH, 17:0, 17:0 cyclo and summed feature 4 in *Rhizobium* sp. (*Hedysarum coronarium*); 15:0 2-OH, 16:0 3-OH, 11 methyl 18:1 ω 8*cis* and summed feature 4 in *R. leguminosarum*; and 15:0 2-OH, 16:0 3-OH and 11 methyl 18:1 ω 8*cis* in *R. etli*.

The fatty acid profile of *Rhizobium* sp. (*Hedysarum coronarium*) was similar to that of *S. meliloti* and *R. leguminosarum*; however, it deviated by containing significantly higher levels of 16:0 3-OH. It could be further distinguished from *S. meliloti* and other members of the genus *Rhizobium* by containing the lowest levels of 20:3 ω 6,9,12*cis* and a moderate concentration of 19:0 cyclo ω 8*cis*. *R. hainanense* was similar to *R. tropici* and *Agrobacterium* biovar 2 in a separate lineage containing 15:0 iso 3-OH which is unique to this group. Although *R. hainanense* was similar to *Agrobacterium* biovar 2, it could be distinguished by the presence of 13:1, unknown (ECL 14-952) and unknown (ECL 11-541). It was notable that *R. hainanense*, *R. tropici* and *Agrobacterium* biovar 2 were the only species that contained 15:0 iso 3-OH. Furthermore, *R. hainanense*, *Agrobacterium* biovar 2, *Agrobacterium* biovar 3 and *R. tropici* all contained 18:1 2-OH at similar levels. *R. gallicum* appeared to be most similar to *R. etli*, but could be distinguished by containing a higher concentration of 16:0 3-OH and a lower concentration of 20:3 ω 6,9,12*cis*. Further discrimination was possible by the presence of 11 methyl 18:1 ω 7*cis* in *R. etli* and not in *R. gallicum*. Analysis of *R. giardinii* revealed significant differences from the other rhizobia. It contained a higher concentration of 16:0, 17:0 cyclo, summed feature 3 and summed feature 4 and the lowest concentration of 19:0 cyclo ω 5*cis* among the rhizobia. It is further distinguished by lacking 16:0 3-OH, a significant fatty acid which contributes to distinguishing the genera *Rhizobium* and *Sinorhizobium*.

***Sinorhizobium*.** Fatty acids common to all species of this genus included 16:0, 18:0, 19:0 cyclo ω 8*cis*, 20:3 ω 6,9,12*cis*, summed feature 3 and summed feature 7. Additional fatty acids detected in individual species included 16:0 3-OH, 17:0, 17:0 cyclo, 19:0 10 methyl and summed feature 4 in *S. meliloti*; 16:0 3-OH, 17:0, 19:0 10 methyl and summed feature 4 in *S. fredii*; 17:0, 19:0 10 methyl and summed feature 4 in *S. kostiense*; 19:0 10 methyl and summed feature 4 in *S. saheli*; and summed feature 4 in *S. teranga*.

Fatty acid patterns in the genus *Sinorhizobium* were similar to those of the genus *Rhizobium*, but differed by generally containing a greater concentration of summed feature 7 and a lower concentration of 19:0 cyclo ω 8*cis* and 16:0 3-OH. Although this relationship holds for most of the species, *S. meliloti* has concentrations of summed feature 7 and 19:0 cyclo ω 8*cis* more characteristic of the genus *Rhizobium*, but contains 16:0 3-OH at a concentration characteristic of the genus *Sinorhizobium*. This suggests that 16:0 3-

OH is an important fatty acid for discriminating between the genera.

Results indicated that fatty acid analysis is in agreement with phylogenetic data that place *S. fredii* into this genus. *S. saheli* was similar to *S. fredii* but differed because it contained a higher concentration of summed feature 3 and a lower concentration of 18:0. Since only six strains were available for analysis, conclusive results were not obtainable. Analysis of *S. teranga* revealed a close similarity to *S. fredii* and *S. saheli*, with only minor differences in the concentration of summed feature 7. *S. kostiense*, *S. arboris* and *S. fredii* appeared to be very similar to one another with insignificant differences in the concentration of 17:0, 19:0 10 methyl and 19:0 cyclo ω 8*cis*. Data collected for this genus revealed a higher level of fatty acid similarity between species than for the other genera evaluated.

***Bradyrhizobium*.** The fatty acids detected in all species of the genus *Bradyrhizobium* were 16:0 and summed feature 7. Additional fatty acids detected in individual species included 16:1 ω 5*cis*, 17:1 ω 8*cis*, 18:0, 11 methyl 18:1 ω 7*cis*, summed feature 4 and 19:0 cyclo ω 8*cis* in *B. japonicum*; 16:1 ω 5*cis*, 17:0, 17:1 ω 6*cis*, 17:1 ω 8*cis* and 19:0 cyclo ω 8*cis* in *B. liaoningense*; 17:0 cyclo, 18:0, 19:0 cyclo ω 8*cis* and summed feature 4 in *B. elkanii* group I; 19:0 cyclo ω 8*cis* and summed feature 4 in *B. elkanii* group II; and 16:1 ω 5*cis* in *Bradyrhizobium* sp. (*Arachis hypogaea*).

Comparisons of bradyrhizobia fatty acid profiles with those of other members of the genera indicate that they possess a unique ratio of fatty acids. Unlike other rhizobia, bradyrhizobia are composed primarily of 16:0 and summed feature 7 that make up 90% of the total fatty acids. Examination of *B. elkanii* indicated that two subgroups exist which were distinguished by the concentration of 19:0 cyclo ω 8*cis* and summed feature 7. These two groups were designated *B. elkanii* fatty acid groups I and II for the purposes of this manuscript. This finding has also been reported elsewhere (Graham *et al.*, 1995). *B. elkanii* could be distinguished from *B. japonicum* by containing a greater concentration of 19:0 cyclo ω 8*cis*. *B. liaoningense* strains could be distinguished from other bradyrhizobia by containing a greater concentration of 16:0 and possessing a concentration of 19:0 cyclo ω 8*cis* and summed feature 7 between that of *B. elkanii* and *B. japonicum*. Strains of *Bradyrhizobium* sp. (*Arachis hypogaea*) were similar to *B. japonicum*, but differed by containing a greater concentration of 16:1 ω 5*cis* and insignificant levels of 19:0 cyclo ω 8*cis*.

Similarity matrix

The data presented in Table 5 indicate the relative similarity between species as determined by the Sherlock MIS cluster analysis software. Values presented in the similarity matrix are computed on the basis of the identification values of all strains. Identi-

Organism		Agrobacterium			Bradyrhizobium					Mesorhizobium							Rhizobium							Sinorhizobium						
		Biovar 1	Biovar 2	Biovar 3	B. elkanii group I	B. elkanii group II	B. japonicum	B. liaoningense	B. sp. (Arachis)	M. ciceri	M. huakuii	M. mediterraneum	M. loti	M. tianshanense group I	M. tianshanense group II	M. plurifarium	R. etli	R. galegae	R. sp. (Hedysarum)	R. hainanense	R. leguminosarum	R. tropici	R. gallicum	R. giardinii	S. fredii	S. meliloti	S. saheli	S. terangae	S. kostiense	S. arboris
Agrobacterium	Biovar 1 Biovar 2 Biovar 3	80.6 64.3 0.7	3.3	13.8													21.0		36.6		35.5		0.1							
Bradyrhizobium	B. elkanii group I B. elkanii group II B. japonicum B. liaoningense B. sp. (Arachis)			73.6 7.7 62.5 2.0 5.1 62.8 27.9 18.2 1.7 12.0 67.3 9.6 51.9										12.6																
Mesorhizobium	M. ciceri M. huakuii M. mediterraneum M. loti M. tianshanense group I M. tianshanense group II M. plurifarium			3.3 0.5 0.1 12.9 29.9 1.0					63.1 9.9 21.9 4.6 63.7 0.5 24.0 11.8 18.1 9.9 4.0 71.5 3.0 22.6 7.7 27.0 16.7 2.7 68.4 3.5 65.6 51.0 74.2																					
Rhizobium	R. etli R. galegae R. sp. (Hedysarum) R. hainanense R. leguminosarum R. tropici R. gallicum R. giardinii	17.8 0.6 6.7 29.0 6.3															54.6 72.1 64.2 1.6 8.5 26.1 23.6 62.9 2.4 0.1 19.3 6.9 7.4 58.3 92.6			34.0 30.8 63.4 62.9 64.5										
Sinorhizobium	S. fredii S. meliloti S. saheli S. terangae S. kostiense S. arboris	0.3 9.2																4.8 1.5 1.0						55.0 5.0 10.4 67.9 2.1 63.2 9.1 11.4 5.3 12.9 52.2 5.0 10.3 32.4 23.9 3.1 51.0 10.5 7.5 32.5 5.1 53.3						

Organism		Agrobacterium			Bradyrhizobium					Mesorhizobium							Rhizobium							Sinorhizobium							
		Biovar 1	Biovar 2	Biovar 3	B. elkanii group I	B. elkanii group II	B. japonicum	B. liaoningense	B. sp. (Arachis)	M. ciceri	M. huakuii	M. mediterraneum	M. loti	M. tianshanense group I	M. tianshanense group II	M. plurifarum	R. etli	R. galegae	R. sp. (Hedysarum)	R. hainanense	R. leguminosarum	R. tropici	R. gallicum	R. giardinii	S. fredii	S. meliloti	S. saheli	S. terangae	S. kostiense	S. arboris	
Agrobacterium	Biovar 1 Biovar 2 Biovar 3	80.6 64.3 0.7	3.3		13.8												21.0		36.6		35.5		0.1								
Bradyrhizobium	B. elkanii group I B. elkanii group II B. japonicum B. liaoningense B. sp. (Arachis)				73.6 7.7 62.5 2.0 5.1 62.8 27.9 18.2 1.7 12.0 67.3 9.6 51.9	32.4 2.0 5.1 62.8 12.0 67.3 9.6 51.9	3.7							12.6	36.5								6.2								
Mesorhizobium	M. ciceri M. huakuii M. mediterraneum M. loti M. tianshanense group I M. tianshanense group II M. plurifarum				3.3 0.5 0.1 12.9 29.9 1.0				63.1 9.9 21.9 4.6 63.7 0.5 24.0 11.8 18.1 9.9 4.0 71.5 3.0 22.6 7.7 27.0 16.7 2.7 68.4 3.5 65.6 51.0 74.2																						
Rhizobium	R. etli R. galegae R. sp. (Hedysarum) R. hainanense R. leguminosarum R. tropici R. gallicum R. giardinii	17.8 0.6 6.7 29.0 6.3															54.6 72.1 64.2 1.6 8.5 26.1 23.6 62.9 2.4 0.1 19.3 6.9 7.4 58.3 92.6			34.0 30.8 63.4 62.9 64.5											
Sinorhizobium	S. fredii S. meliloti S. saheli S. terangae S. kostiense S. arboris	0.3 9.2																4.8 1.5 1.0						55.0 5.0 10.4 67.9 2.1 63.2 9.1 11.4 5.3 12.9 52.2 5.0 10.3 32.4 23.9 3.1 51.0 10.5 7.5 32.5 5.1 53.3							

fication values are not included in this manuscript due to the large quantity of data they encompass. Proper evaluation of the table is done by inspecting in a vertical fashion.

Our data suggested that a group with a similarity value of less than 55% to itself is difficult or impossible to distinguish from the next most similar group using this method. When this value was observed in conjunction with a value of 27% or greater to another group, it was not possible to discriminate the groups. However, it was notable that discrepancies in values could be observed for species of which less than twenty strains were analysed. An example of this can be observed when comparing the similarity values of *M. ciceri* (9 strains examined) with *M. loti* (17 strains examined). When comparing the vertical column value for *M. ciceri* to the horizontal value of *M. loti*, a value of 27% is observed, but when comparing the vertical column value of *M. loti* to the horizontal value of *M. ciceri*, a 4.6% similarity is observed. This unfortunate situation is a shortcoming of the MIS software when not enough strains of each species are used to build a species fatty acid profile. Although this situation could have been avoided by evaluating more strains, they were not available when this study was conducted.

Similarity values for biovars belonging to the genus *Agrobacterium* indicated that *Agrobacterium* biovar 1 has similarity to *R. galegae*, and *Agrobacterium* biovar 2 has similarity to *R. tropici*. *Agrobacterium* biovar 3 has low similarity with *Agrobacterium* biovar 1, *R. giardinii* and *S. fredii*. Similarity values for members of the genus *Bradyrhizobium* indicate that *B. elkanii* fatty acid group I has similarity to *Agrobacterium* biovar 1, *B. elkanii* fatty acid group II and *B. liaoningense*. *B. elkanii* fatty acid group II has a high similarity to *B. elkanii* fatty acid group I and low similarity to *B. japonicum*. *B. liaoningense* has similarity to *B. japonicum* and little similarity with *B. elkanii* and *M. plurifarium*. *Bradyrhizobium* sp. (*Arachis hypogaea*) has a strong similarity to *M. tianshanense* fatty acid group II and *B. japonicum*. Interestingly, the similarity between *Bradyrhizobium* sp. (*Arachis hypogaea*) and *M. tianshanense* fatty acid group II is so high that these organisms are undistinguishable. Further evaluation is needed to support the classification of strains belonging to *M. tianshanense* fatty acid group II.

Similarity values for species belonging to the genus *Mesorhizobium* indicate that *M. ciceri* is similar to *M. loti* and less similar to *M. mediterraneum*. Likewise, *M. mediterraneum* shows similarity with *M. ciceri* and little similarity with *M. loti* and *M. plurifarium*. *M. tianshanense* fatty acid group I shows similarity to *M. mediterraneum* and less to *M. huakuii*. *M. plurifarium* appears to be unique, with similarity to *M. huakuii* and *M. mediterraneum*.

Results for the genus *Rhizobium* indicated a high similarity between *R. etli* and *R. leguminosarum*. This

is not unexpected since it is known that these species are closely related (Segovia *et al.*, 1993). *Rhizobium* sp. (*Hedysarum coronarium*) has a similarity with *R. leguminosarum* and *S. meliloti*, and less with *R. gallicum*. *R. hainanense* has similarity with *Agrobacterium* biovar 2, whilst *R. gallicum* has similarity with *R. etli* and less with *Rhizobium* sp. (*Hedysarum coronarium*) and *R. leguminosarum*. *R. giardinii* has little similarity with *Agrobacterium* biovar 3 and appears to be unique.

Similarities among the sinorhizobia appeared to be greater than for other genera. These data showed that *S. fredii* is similar to *S. kostiense* and *S. meliloti*. *S. meliloti* has similarity with both *Rhizobium* sp. (*Hedysarum coronarium*) and *S. arboris*. *S. saheli* has similarity with both *S. terangae* and *S. kostiense*, whilst *S. terangae* is most similar to *S. arboris*. *S. kostiense* has similarity with *S. fredii* and the two are indistinguishable using this method. Similarity values for *S. arboris* seem to be evenly weighted for all the other species belonging to this genus.

3-D principal component plot

The 3-D principal component plot shown in Fig. 1 was assembled from coordinates generated by from 2-D principal component data of the Sherlock MIS software. This type of plot is very useful when evaluating species relatedness based on clustering. Although this plot appears to reveal the separation of many of the groups, it should be noted that the principal components are composed of a combination of several fatty acids and do not take into account all fatty acids. Unfortunately, the selection of fatty acids used in the multivariate calculations to generate the plot are determined by the MIS software and cannot be modified by the investigator without changes to the source code. Although the 3-D plot does not take into account all fatty acids detected, it is effective at clustering similar groups and separating groups that exhibit a significant degree of dissimilarity.

This plot indicates that the species belonging to the genus *Agrobacterium* are distributed throughout the *Agrobacterium*–*Rhizobium*–*Sinorhizobium* super-cluster. The position of *Agrobacterium* biovar 1 is separate from other groups and related to *R. galegae*. *Agrobacterium* biovar 2 is close to *R. hainanense* and *R. tropici*, whilst *Agrobacterium* biovar 3 clusters near *R. giardinii*, *R. fredii*, *S. saheli* and *S. arboris*. The genus *Mesorhizobium* is separate in this plot from other genera and is composed of three clusters that contain *M. ciceri* and *M. mediterraneum*, *M. tianshanense* fatty acid group I and *M. plurifarium*, and *M. huakuii* and *M. loti*. Clustering of species belonging to the genus *Rhizobium* indicates that *R. etli*, *R. gallicum*, *R. leguminosarum* and *Rhizobium* sp. (*Hedysarum coronarium*) make up a single cluster. The positions of *R. hainanense* and *R. tropici* are near that of *Agrobacterium* biovar 2, whilst *R. galegae* clusters

Fig. 1. 3-D plot of fatty acid principal components for all species evaluated in this study. A1, *Agrobacterium* biovar 1; A2, *Agrobacterium* biovar 2; A3, *Agrobacterium* biovar 3; BE1, *Bradyrhizobium elkanii* group I; BE2, *Bradyrhizobium elkanii* group II; BL, *Bradyrhizobium liaoningense*; BP, *Bradyrhizobium* sp. (*Arachis*); CER, *Mesorhizobium ciceri*; HUA, *Mesorhizobium huakuii*; LOT, *Mesorhizobium loti*; MED, *Mesorhizobium mediterraneum*; MT1, *Mesorhizobium tianshanense* group I; MT2, *Mesorhizobium tianshanense* group II; CU, *Mesorhizobium plurifarium*; ETL, *Rhizobium etli*; GAL, *Rhizobium galegae*; HED, *Rhizobium* sp. (*Hedysarum*); HAI, *Rhizobium hainanense*; LEG, *Rhizobium leguminosarum*; TRP, *Rhizobium tropici*; 602, *Rhizobium gallicum*; 152, *Rhizobium giardinii*; FRE, *Sinorhizobium fredii*; MEL, *Sinorhizobium meliloti*; SAH, *Sinorhizobium saheli*; TER, *Sinorhizobium terangae*; C8, *Sinorhizobium kostense*; C11, *Sinorhizobium arboris*.

near *Agrobacterium* biovar 1 and *Agrobacterium* biovar 2. The position of *R. giardinii* is unique, but appears to cluster near *Agrobacterium* biovar 3. In the genus *Sinorhizobium*, *S. meliloti* clusters near *Rhizobium* sp. (*Hedysarum coronarium*), *S. fredii* clusters with *S. arboris*, *R. etli* and *R. gallicum*, and *S. saheli* clusters with *S. kostense* and *R. giardinii*. The position of *S. terangae* appears separate from other members of the genus.

The position of the genus *Bradyrhizobium* on this plot is quite remote compared to the other genera. This is not surprising considering that the genus *Bradyrhizobium* is vastly different from the other rhizobia. In this plot, *B. japonicum* and *B. elkanii* fatty acid types I and II are separate, but the positions of *B. liaoningense*,

Bradyrhizobium sp. (*Arachis hypogaea*) and *R. tianshanense* group II are close to *B. japonicum*.

DISCUSSION

In this study, the Sherlock Microbial Identification System (MIS) was used to collect cellular fatty acid data and to evaluate relationships between species belonging to the genera *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. Although previous studies had examined several species belonging to these genera, they did not involve such a large number of strains from so many species as here. In this study, we have included some previous data which have been amended to include the

compound 11 methyl 18:1 ω 7*cis*, which had been previously detected but unidentified. This additional piece of data slightly modifies data reported in our previous studies (Jarvis *et al.*, 1996; Jarvis & Tighe, 1994). This discovery affects the total quantity of fatty acids in each strain and the overall relationships between strains. Considering the new compounds and using the data collected, we have assembled a table of fatty acids, a similarity matrix and a 3-D principal component plot for all the species examined.

Results collected for the genus *Agrobacterium* revealed three clusters corresponding to *Agrobacterium* biovar 1, *Agrobacterium* biovar 2 and *Agrobacterium* biovar 3. In agreement with phylogenetic data, *Agrobacterium* biovar 1 shows similarity to *R. galegae* (Terefework *et al.*, 1998) whilst *Agrobacterium* biovar 2 has similarity to *R. tropici* and *R. hainanense* (Chen *et al.*, 1997). *Agrobacterium* biovar 3 has a unique ratio of fatty acids with very little similarity to the genus *Rhizobium*. Data for *A. rubi* (DNA group TR2) were not included in this study because only two strains were available for analysis.

Evaluation of the genus *Bradyrhizobium* revealed significant fatty acid differences from all other rhizobia studied. This is not surprising as research has shown that bradyrhizobia are significantly different from the other rhizobia and agrobacteria (Jordan, 1982). Our results indicate that *B. japonicum* and *B. elkanii* are readily distinguished from one another, and that two fatty acid subgroups of *B. elkanii* were observed. *B. liaoningense* has been described as an extra slow-growing (ESG) species with similarities to *B. japonicum* (Xu *et al.*, 1995). This is supported by cellular fatty acid data and only minor differences in two fatty acids have been observed between the two species. Strains of *Bradyrhizobium* sp. (*Arachis hypogaea*) were also similar to *B. japonicum* but because of such similar fatty acid profiles, reliable discrimination was not possible.

The genus *Mesorhizobium* was established to accommodate organisms belonging to the *R. loti*–*R. huakuii* cluster (Jarvis *et al.*, 1997). Fatty acid analysis of this genus showed clear discrimination between the species. The phylogenetically defined species evaluated in this study include *M. huakuii*, *M. loti*, *M. ciceri*, *M. mediterraneum*, *M. tianshanense* and *M. plurifarum*. Although fatty acid analysis has previously been performed on several of the species belonging to this group, an important discriminating fatty acid has since been identified as 11 methyl 18:1 ω 7*cis* and included in this study. This compound is useful for discriminating between species belonging to the genus *Mesorhizobium* and for discrimination from other genera.

The similarities observed between *M. ciceri*, *M. loti* and *M. mediterraneum* support the phylogenetic data reported between these species (Jarvis *et al.*, 1997). *M. huakuii* strains formed a separate cluster with similarity to the *M. loti* cluster. *M. plurifarum* is similar to *M. tianshanense* fatty acid group I and less similar to *M.*

loti. Although this dissimilarity is not evident in the similarity matrix or 3-D plot, it can be detected when evaluating the fatty acid data in Table 1. This finding supports other studies that define *M. plurifarum* as a new species (de Lajudie *et al.*, 1998b).

Analysis of strains received as *M. tianshanense* revealed two distinct groups with different fatty acid profiles. These have been designated fatty acid groups I and II for the purposes of this investigation. Fatty acid group I appears similar to *M. huakuii* and *M. plurifarum* and these data support the earlier designation of these strains as a new species belonging to the genus *Mesorhizobium* based on phylogenetic studies (Chen *et al.*, 1995; Jarvis *et al.*, 1997). The four strains constituting fatty acid group II appear identical to *Bradyrhizobium* sp. (*Arachis hypogaea*) and could not be distinguished using fatty acid analysis. This suggests that the original classification of these strains may be questionable.

Although the fatty acid characteristics and relationships of the genus *Rhizobium* have been previously described, their relationship with new species belonging to the genus *Sinorhizobium* has not been addressed. The clustering of rhizobial species based on fatty acid analysis is unusual because several separate clusters are observed. *R. etli*, *R. gallicum*, *Rhizobium* sp. (*Hedysarum coronarium*) and *R. leguminosarum* form one cluster. Within this cluster, *R. etli* with *R. leguminosarum* and *R. gallicum* form a subcluster with closer fatty acid similarity. The fatty acid profiles of these species permit effective discrimination between *R. gallicum* and *R. etli*, but are less able to discriminate between *R. leguminosarum* and *R. etli*.

Fatty acid analysis suggests that *Rhizobium* sp. (*Hedysarum coronarium*) has a unique fatty acid pattern with close similarity to *S. meliloti*. However, these two organisms can be distinguished based on the concentration of 20:3 ω 6,9,12*cis* and 16:0 3-OH. *R. hainanense*, *R. tropici* and *R. galegae* form a separate fatty acid cluster with comparatively little similarity to other species belonging to the genus *Rhizobium*. Fatty acid data collected for *R. hainanense*, *R. tropici* and *Agrobacterium* biovar 2 are in broad agreement with phylogenetic data which indicates similarity of the species (Lindström *et al.*, 1995). Nonetheless, fatty acid analysis is capable of discriminating between the species. Phylogenetic data indicate that *R. galegae* and *R. giardinii* have similarities to members of the genus *Agrobacterium* (Amarger *et al.*, 1997). These reports support our findings that indicate *R. galegae* is similar to *Agrobacterium* biovar 1, and *R. giardinii* has little similarity to *Agrobacterium* biovar 3. In both cases, fatty acid analysis is able to discriminate between these species.

The genus *Sinorhizobium* was established in 1988 to accommodate the fast-growing soybean rhizobia (Chen *et al.*, 1988). Currently this genus contains eight named species, of which we have examined six. The similarities observed among species belonging to this

genus using fatty acid analysis are much greater than those seen in other genera evaluated. Interestingly, our results indicate a discriminatory role for the fatty acid 16:0 3-OH. Organisms belonging to the genus *Sinorhizobium* contain low levels of this compound, whilst members of the genus *Rhizobium* contain significantly higher levels. This suggests that 16:0 3-OH is important for discriminating between the two genera and can be observed in the case of *S. meliloti* and *Rhizobium* sp. (*Hedysarum coronarium*). These two organisms possess a very similar fatty acid profile and cluster together in the 3-D plot, but the concentration of 16:0 3-OH serves to place them in their respective genera.

Although the fatty acid data we have collected for the genera *Agrobacterium*, *Rhizobium* and *Bradyrhizobium* can be compared with that of other researchers (Yokota & Sakane, 1991; Yokota *et al.*, 1993), they evaluated fewer strains and species and used different strains, growth media and analytical methods to detect the fatty acids. This resulted in fatty acid data that was compositionally different from that observed in this study using the Sherlock MIS. However, the data collected by both groups are in broad agreement.

Although the sole use of fatty acid data to compare one species with another is not typically employed for the purposes of taxonomy, it can be used in conjunction with other taxonomic information such as phylogenetic data. Similarities derived from fatty acid analysis are in broad agreement with 16S rDNA results and appear to accurately distinguish between most species. The Sherlock MIS is an attractive tool in phenotypic taxonomy because it can rapidly assay a large number of unknown strains and correlates well with genomic data.

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REFERENCES

- Amarger, N., Macheret, V. & Laguerre, G. (1997). *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov. from *Phaseolus vulgaris* nodules. *Int J Syst Bacteriol* **47**, 996–1006.
- van Berkum, P., Beyene, D., Bao, G., Campbell, A. & Eardly, B. D. (1998). *Rhizobium mongolense* sp. nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.) Ledebour]. *Int J Syst Bacteriol* **48**, 13–22.
- Bradbury, J. F. (1986). *Agrobacterium*. In *A Guide to Plant Pathogenic Bacteria*, pp. 6–15. Farnham Royal: CAB International Mycological Institute.
- Casella, S., Shapleigh, J. P. & Payne, W. J. (1986). Nitrate reduction in “*Rhizobium hedysari*” strain HCNT 1. *Arch Microbiol* **146**, 233–238.
- Chen, W. X., Yan, G. H. & Li, J. L. (1988). Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *Int J Syst Bacteriol* **38**, 392–397.
- Chen, W. X., Wang, E. T., Li, Y. B., Chen, X. Q. & Li, Y. (1995). Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People's Republic of China. *Int J Syst Bacteriol* **45**, 153–159.
- Chen, W. X., Tan, Z. Y., Gao, J. L., Li, Y. & Wang, E. T. (1997). *Rhizobium hainanense* sp. nov., isolated from tropical legumes. *Int J Syst Bacteriol* **47**, 870–873.
- Graham, P. H., Sadowsky, M. J., Tighe, S. W., Thompson, J. A., Date, R. A., Howieson, J. G. & Thomson, R. (1995). Differences among strains of *Bradyrhizobium* in fatty acid-methyl ester analysis. *Can J Microbiol* **41**, 1038–1042.
- Jarvis, B. D. W. & Tighe, S. W. (1994). Rapid identification of *Rhizobium* species based on cellular fatty acid analysis. *Plant Soil* **161**, 31–41.
- Jarvis, B. D. W., Downer, H. L. & Young, J. P. W. (1992). Phylogeny of fast-growing soybean-nodulating rhizobia supports synonymy of *Sinorhizobium* and *Rhizobium* and assignment to *Rhizobium fredii*. *Int J Syst Bacteriol* **42**, 93–96.
- Jarvis, B. D. W., Sivakumaran, S., Tighe, S. W. & Gillis, M. (1996). Identification of *Agrobacterium* and *Rhizobium* species based on cellular fatty acid composition. *Plant Soil* **184**, 143–158.
- Jarvis, B. D. W., Van Berkum, P., Chen, W. X., Nour, S. M., Fernandez, M. P., Cleyet-Marel, J. C. & Gillis, M. (1997). Transfer of *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. *Int J Syst Bacteriol* **47**, 895–898.
- Jordan, D. C. (1982). Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing root nodule bacteria from leguminous plants. *Int J Syst Bacteriol* **32**, 136–139.
- Jordan, D. C. (1984). Family III *Rhizobiaceae*. *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 234–244. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Keyser, H. H., Bohlool, B. B., Hu, B. & Weber, D. (1982). Fast growing rhizobia isolated from root nodules of soybean. *Science* **215**, 1631–1632.
- de Lajudie, P., Willems, A., Pot, B. & 7 other authors (1994). Polyphasic taxonomy of rhizobia: emendation of the genus

- Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov. and *Sinorhizobium teranga* sp. nov. *Int J Syst Bacteriol* **44**, 715–733.
- de Lajudie, P., Laurent-Fulele, E., Willems, A., Torck, U., Coopman, R., Collins, M. D., Kersters, K., Dreyfus, B. & Gillis, M. (1998a). *Allorhizobium undicola* gen. nov., sp. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. *Int J Syst Bacteriol* **48**, 1277–1290.
- de Lajudie, P., Willems, A., Nick, G. & 9 other authors (1998b). Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarum* sp. nov. *Int J Syst Bacteriol* **48**, 369–382.
- Lindström, K., Van Berkum, P., Gillis, M., Martinez, E., Novikova, N. & Jarvis, B. D. W. (1995). *Nitrogen Fixation: Fundamentals and Applications. Report from the Roundtable on Rhizobium Taxonomy*, pp. 807–810. Edited by I. A. Tikhonovich, N. A. Provorov, V. I. Romanov & W. E. Newton. Dordrecht: Kluwer.
- Long, S. (1989). *Rhizobium*–legume nodulation: life together in the underground. *Cell* **56**, 203–214.
- Lynch, J. M. (1976). Products of soil microorganisms in relation to plant growth. *Crit Rev Microbiol* **5**, 67–107.
- Nick, G., de Lajudie, P., Eardly, B. D., Suomalainen, S., Paulin, L., Zhang, X., Gillis, M. & Lindström, K. (1999). *Sinorhizobium arboris* sp. nov. and *Sinorhizobium kostiense* sp. nov., isolated from leguminous trees in Sudan and Kenya. *Int J Syst Bacteriol* **49**, 1359–1368.
- Orgambide, G. G., Reusch, R. N. & Dazzo, F. B. (1993). Methoxylated fatty acids reported in *Rhizobium* isolates arise from chemical alterations of common fatty acids upon acid-catalyzed transesterification procedures. *J Bacteriol* **175**, 4922–4926.
- Rome, S., Fernandez, M. P., Brunel, B., Normand, P. & Cleyet-Marel, J. C. (1996). *Sinorhizobium medicae* sp. nov., isolated from annual *Medicago* spp. *Int J Syst Bacteriol* **46**, 972–980.
- Sasser, M. (1990). Identification of bacteria by gas chromatography of cellular fatty acids. MIDI Technical Note **101**. Microbial ID, Inc., Newark, DE, USA.
- Sawada, H., Takikawa, Y. & Ieki, H. (1992). Fatty acid methyl ester profiles of the genus *Agrobacterium*. *Ann Phytopathol Soc Jpn* **58**, 46–51.
- Segovia, L., Young, J. P. W. & Martinez-Romero, E. (1993). Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I strains as *Rhizobium etli* sp. nov. *Int J Syst Bacteriol* **43**, 374–377.
- Terefework, Z., Nick, G., Suomalainen, S., Paulin, L. & Lindström, K. (1998). Phylogeny of *Rhizobium galegae* with respect to other rhizobia and agrobacteria. *Int J Syst Bacteriol* **48**, 349–356.
- Wang, E. T., Van Berkum, P., Beyene, D., Sui, X. H., Chen, W. X. & Martinez-Romero, E. (1998). *Rhizobium huautlense* sp. nov., a symbiont of *Sesbania herbacea* that has a close relationship with *Rhizobium galegae*. *Int J Syst Bacteriol* **48**, 687–699.
- Wang, E. T., Van Berkum, P., Sui, X. H., Beyene, D., Chen, W. X. & Martinez-Romero, E. (1999). Diversity of rhizobia associated with *Amorpha fruticosa* isolated from Chinese soils and description of *Mesorhizobium amorphae* sp. nov. *Int J Syst Bacteriol* **49**, 51–65.
- Willems, A. & Collins, M. D. (1993). Phylogenetic analysis of rhizobia and agrobacteria based on 16S ribosomal DNA sequences. *Int J Syst Bacteriol* **43**, 305–313.
- Xu, L. M., Ge, C., Cui, Z., Li, J. & Fan, H. (1995). *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. *Int J Syst Bacteriol* **45**, 706–711.
- Yokota, A. & Sakane, T. (1991). Taxonomic significance of fatty acid compositions in whole cells and lipopolysaccharides in *Rhizobiaceae*. *Inst Ferment Res Commun* **15**, 57–75.
- Yokota, A., Sakane, T., Ophel, K. & Sawada, H. (1993). Further studies on the cell fatty acid composition of *Rhizobium* and *Agrobacterium* species. *Inst Ferment Res Commun* **16**, 86–94.
- Young, J. P. W. & Haukka, K. E. (1996). Diversity and phylogeny of rhizobia. *New Phytol* **133**, 87–94.
- Zhang, X., Nick, G., Kaijalainen, S., Terefework, Z., Paulin, L., Tighe, S. W., Graham, P. H. & Lindström, K. (1999). Phylogeny and diversity of *Bradyrhizobium* strains isolated from the root nodules of peanut (*Arachis hypogaea*) in Sichuan, China. *Syst Appl Microbiol* **22**, 378–386.