NOTE

Phylogenetic relationships of three bacterial strains isolated from the pasture legume *Biserrula pelecinus* L.

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Three bacterial strains (WSM 1283, WSM 1284, WSM 1497) isolated from root nodules of the pasture legume *Biserrula pelecinus* L. growing in Morocco, Italy and Greece, respectively, were studied in order to determine their phylogenetic relationship to the other members of the family *Rhizobiaceae*. A polyphasic approach, which included analyses of morphological and physiological characteristics, plasmid profiles, symbiotic performance and 16S rRNA gene sequencing, indicated that these strains belong to the genus *Mesorhizobium*.

Keywords: *Biserrula*, *Mesorhizobium*, pasture legumes, rhizobia, phylogenetic relationships

Biserrula pelecinus L. was recently introduced from the Mediterranean basin to Australian agriculture as an alternative pasture legume for acid soils (Howieson *et al.*, 1995) because of its many agronomic attributes (Howieson *et al.*, 2000; Loi *et al.*, 1997). Preliminary data indicate considerable specificity in the symbiotic relationship between *B. pelecinus* and its nodule bacteria (Howieson *et al.*, 1995). Strains isolated from *B. pelecinus* did not nodulate common agricultural legume species and *Rhizobium leguminosarum* bv. *trifolii*, *R. leguminosarum* bv. *viciae*, *Sinorhizobium meliloti* and strains of *Bradyrhizobium* spp. did not nodulate *B. pelecinus* (Howieson *et al.*, 1995).

The taxonomy of the legume root-nodule bacteria follows a polyphasic approach that separates the *Rhizobiaceae* into six genera based on differences in their 16S rRNA sequences, host specificity for nodulation, presence of plasmids, location of the symbiotic genes and physiological characteristics (Graham *et al.*, 1991; Martínez-Romero *et al.*, 2000; Young, 1996). The stem-nodulating bacteria are in the genus *Azorhizobium* (Dreyfus *et al.*, 1988) and slow-growing rhizobia are in the genus *Bradyrhizobium*, while the

fast-growing rhizobia are distributed across the other four genera, *Allorhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* (Jordan, 1984; Lindström *et al.*, 1998; Young, 1996, 2000). Rhizobia isolated from *B. pelecinus* have not been studied systematically and our aim was to investigate the phylogenetic relationship of these bacteria through a polyphasic approach.

Three strains (WSM 1283, WSM 1284 and WSM 1497) isolated from nodules on roots of *B. pelecinus* growing in Morocco, Italy and Greece, respectively (Table 1), were grown on yeast mannitol agar (YMA; Vincent, 1970) or 1/2 lupin agar (Howieson *et al.*, 1988) at 28 °C for observation of colony morphology and in yeast mannitol broth (YMB; Vincent, 1970) for physiological experiments. The strains were moderately fast-growing, forming 2–4 mm diameter colonies within 4–5 d on YMA, and having a mean generation time of 4 h when grown in YMB at 28 °C. Colonies on YMA were white-opaque, slightly domed, moderately mucoid with smooth margins. The strains grew on YMA containing 1.5% (w/v) NaCl, but not with 2.0% (w/v) NaCl. YMA buffered with homopiperazine-*N,N'*-bis-2-(ethanesulfonic acid) (HOMOPIPES) (pH 4.0–5.0), MES (pH 5.0–6.0) or HEPES (pH 6.5–8.0) was used to assess the effect of pH on growth. The strains grew well on YMA buffered between pH 5.5 and 8.0 with HEPES or MES and showed considerable growth on YMA buffered at pH 5.0 with HOMOPIPES. There was no growth at or below pH 4.5.

Abbreviations: HOMOPIPES, homopiperazine-*N,N'*-bis-2-(ethanesulfonic acid); YMA, yeast/mannitol agar; YMB, yeast/mannitol broth.

The GenBank accession numbers for the 16S rRNA gene sequences of strains WSM 1284, WSM 1283 and WSM 1497 are AF178962, AF178963 and AF178964.

Table 1. Origin of isolates

All strains were obtained from the Western Australian Soil Microbiology (WSM) culture collection, Centre for *Rhizobium* Studies, Murdoch University, Murdoch, Western Australia. Data on the location of isolation (including altitude and soil type and pH) were taken from Howieson *et al.* (1995). Data on host morphology were taken from Loi *et al.* (1997).

Strain	Location	Host morphology	Altitude	Soil type	Soil pH	Collector(s)
WSM 1283	Oulmes, Morocco	Blue flowers, small seeds	1100 m	Sand	6.0	J. G. Howieson
WSM 1284	Siniscola, Sardinia, Italy	Blue flowers, small seeds	30 m	Sand	6.2	J. G. Howieson
WSM 1497	Mykonos, Greece	White flowers, large seeds	30 m	Sandy loam	8.0	S. Carr and B. Nutt

Fig. 1. Electron micrograph of isolate WSM 1284. Cells are rod-shaped with dimensions of $0.5 \times 1.0 \mu\text{m}$ and a single polar flagellum. Bar, $1 \mu\text{m}$.

Morphological characteristics were observed using the techniques described by Glauret (1975) and Spurr (1969). Cells of the three strains were Gram-negative rods with dimensions of $0.5 \times 1 \mu\text{m}$ and, when grown in tryptone/yeast extract broth for 4 d at 28°C , they contained granules of poly- β -hydroxybutyrate. Cells of all three strains possessed a polar or subpolar flagellum (Fig. 1). Bacteroids in root nodules on *B. pelecinus* were pleomorphic. Eckhardt gel electrophoresis was used to mobilize and visualize plasmids (Eckhardt, 1978) and a single plasmid of approximately 500 kb was present in all three strains.

API galleries (API 50CH and API 50CHE/B medium; bioMérieux) were used to determine carbohydrate utilization profiles. The three strains did not utilize α -methyl D-mannoside, α -methyl D-glucoside, N-acetylglucosamine, amygdalin, inulin, melezitose, glycogen, xylitol, gluconate, 2-ketogluconate or 5-ketogluconate. Strains WSM 1283 and WSM 1284 utilized

L-sorbose but WSM 1497 did not. Only WSM 1284 utilized arbutin and salicin. Strain WSM 1283 did not utilize D-raffinose, while only WSM 1497 utilized starch. The three strains utilized all the other carbohydrates provided in the API 50CH galleries.

Symbiotic interactions with *Lotus corniculatus*, *Lotus uliginosus*, *Lotus ornithopodioides*, *Cicer arietinum* L. cv. Sona, *C. arietinum* L. cv. Kaniva and *Dorycnium hirsutum* were examined using a sand-pot culture method (Howieson *et al.*, 1995). Nodule occupancy was confirmed through randomly amplified polymorphic DNA PCR with the primer RPO1 (Richardson *et al.*, 1995). Strains WSM 1283, WSM 1284 and WSM 1497 formed effective root nodules on *B. pelecinus*. Strain WSM 1284 has a broad host range and formed root nodules on *D. hirsutum*, *L. corniculatus* and *L. ornithopodioides*. Neither WSM 1283 nor WSM 1497 nodulated these species and none of the three strains nodulated *C. arietinum*.

The methods described by Yanagi & Yamasato (1993) were used to sequence the 16S rRNA genes. Initially, a BLAST search was used to find close relations through sequence similarity. The sequence data were analysed and a phylogenetic tree (Fig. 2) was derived as described by Yanagi & Yamasato (1993) and Young (1996). A total of 1400 bases from each sequence were used for comparison and also to develop the phylogenetic tree. A BLAST similarity search revealed that the sequence of strain WSM 1283 shared 98% identity with those of WSM 1284 and WSM 1495 while the latter two strains shared 99% sequence identity. All three isolates shared 99% identity with sequences from *Mesorhizobium loti* and *Mesorhizobium ciceri*. The distance matrix obtained using nucleotide substitution rates (K_{nuc} values) displayed the following values between the biserrula strains and representative members of the other genera: *Bradyrhizobium*, > 11 ; *Azorhizobium*, > 10 ; *Agrobacterium*, > 7 ; *Rhizobium*, > 7 ; *Sinorhizobium*, > 4 ; *Mesorhizobium*, < 2.6 . Therefore, the phylogenetic tree derived from these K_{nuc} values clustered the biserrula isolates among the members of *Mesorhizobium* (Fig. 2). The K_{nuc} values were extremely small between the biserrula bacteria and both *M. loti* (< 0.9) and *M. ciceri* (< 0.7). These values are evidence of a common ancestor for these three groups of bacteria and of the recent divergence of the biserrula isolates from *M. ciceri* and *M. loti*.

Fig. 2. Phylogenetic relationships of the three biserrula isolates (WSM 1283, WSM 1284, WSM 1497) within the family Rhizobiaceae based upon aligned sequences of the small subunit rRNA gene (16S rRNA). Kimura's two-parameter distances were derived from the aligned sequences to construct an unrooted tree using the neighbour-joining method. The sequences used were derived from the type strain wherever possible.

Although the presence of plasmids is not very common in *Mesorhizobium*, especially in *M. loti* (Young, 2000), members of *Mesorhizobium amorphae* carry up to four plasmids, including one of 930 kb (Wang *et al.*, 1999). It is noteworthy that the symbiotic genes of *M. amorphae* are present on this large plasmid whereas, in *M. loti*, the symbiotic genes are present on the chromosome, in a special region called the 'symbiosis island' (Ronson *et al.*, 2000). The investigation of the location of the symbiotic genes in biserrula organisms will contribute to their distinction from the other members of the genus *Mesorhizobium*, especially *M. loti*.

In conclusion, data on the morphology, physiology, 16S rRNA sequences and symbiotic performance of strains WSM 1283, WSM 1284 and WSM 1497, isolated from nodules collected from *B. pelecinus* in Morocco, Italy and Greece, respectively, indicate they belong to the genus *Mesorhizobium* in the family Rhizobiaceae. According to Jarvis *et al.* (1997), the name *Mesorhizobium* is given to bacteria that have a growth rate that is intermediate between those of typical fast growers and typical slow growers. These authors also reported that the presence of polar or subpolar flagella is a characteristic common to the members of the genus *Mesorhizobium*. The flagellation and growth rate of the biserrula isolates are therefore morphological traits that are shared with members of *Mesorhizobium*. As the biserrula isolates grew on disaccharides, they can be separated from the genus *Bradyrhizobium* (Jordan, 1984). Unlike *R. leguminosarum* and *S. meliloti*, the biserrula isolates did not utilize xylitol. Similarly the biserrula isolates utilized D-fucose and erythritol, whereas *R. leguminosarum*

does not. Hence, the carbohydrate utilization patterns of the three biserrula isolates are closer to those of *M. loti* than to those of *Sinorhizobium* or *Rhizobium* species. The plasmid profiles and 16S rRNA sequence-based clustering patterns further confirm that these three bacterial strains belong to the genus *Mesorhizobium*.

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