Phylogenetic relationships of the genera *Stella*, *Labrys* and *Angulomicrobium* within the ‘*Alphaproteobacteria*’ and description of *Angulomicrobium amanitiforme* sp. nov.

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The unusually shaped bacteria of the genera *Stella*, *Labrys* and *Angulomicrobium* have been described based on their cell morphology and biochemistry. However, their phylogenetic relationships remain unresolved. An earlier study that was based on SS rRNA gene sequences placed the genus *Stella* within the ‘*Alphaproteobacteria*’. In the present report, polar lipids and 16S rRNA genes of the type strains of the two species in the genus *Stella*, *Stella humosa* DSM 5900T and *Stella vacuolata* DSM 5901T, are studied, as well as the type strains of the monospecific genera *Labrys* (*Labrys monachus* VKM B-1479T) and *Angulomicrobium* (*Angulomicrobium tetraedrale* DSM 5895T). It was found that the genus *Stella* belongs to the order *Rhodospirillales* in the family *Rhodospirillaceae*, and not to the *Acetobacteraceae*. Whilst the position of the genus *Angulomicrobium* in the family *Hyphomicrobiaceae* was confirmed, the genus *Labrys* could not be placed into any known family, but was adjacent to the family ‘*Beijerinckiaceae*’. In addition, data were obtained for strain VKM B-1336, which was shown not to belong to the genus *Angulomicrobium*, and strain NCIMB 1785T (=DSM 15561T), for which the name *Angulomicrobium amanitiforme* sp. nov. is proposed.

INTRODUCTION

Star-shaped cells have been observed in various aquatic environments and soil samples since 1966 (Hirsch & Schlesner, 1981). The first pure culture of star-shaped bacteria was obtained by Vasilyeva (1970) and was designated strain AUCM B-1137T. Vasilyeva (1985) reported ten additional strains of star-shaped bacteria that had been isolated from soil, compost, horse manure, sewage sludge and marine sediment and described two of these strains as monotypic species, *Stella humosa* AUCM B-1137T (=DSM 5900T) and *Stella vacuolata* INMI 229T (=DSM 5901T). DNA–DNA reassociation experiments showed low DNA–DNA similarity between *S. humosa* DSM 5900T and *S. vacuolata* DSM 5901T (Reimer & Schlesner, 1989). These authors described the isolation of 11 additional strains of star-shaped bacteria from a variety of aquatic habitats and showed, by DNA–DNA hybridization experiments, that the two *Stella* species and the 11 isolates could be divided into at least five distinct groups. Their data suggested that, among the strains investigated, *S. humosa* DSM 5900T and *S. vacuolata* DSM 5901T were genetically the most remotely related, with the 11 additional strains being more or less closely related to one of the two type strains. In spite of *S. humosa* DSM 5900T being one of the first strains for which the 16S rRNA gene was sequenced (Schlesner et al., 1990), to our knowledge, no 16S rRNA gene sequence data have been published so far and the affiliation of the genus *Stella* within the ‘*Alphaproteobacteria*’ has only been deduced from 5S rRNA gene sequence comparisons (Bomar & Stackebrandt, 1987) and 16S rRNA cataloguing (Fischer et al., 1985). The pioneering 5S rRNA study of Bomar & Stackebrandt (1985) placed *S. humosa* within the ‘*Alphaproteobacteria*’, but the limited dataset available at that time only allowed comparison with very few other genera.

In 1984, Vasil’eva and Semenov described a strain of budding prosthecate bacteria that was isolated from silt of Lake Mustiäär in the former Estonian SSR (Vasil’eva & Semenov,
1984). Based on radial cell symmetry, multiplication by budding and the presence of prosthecae, the authors placed this strain, VKM B-1479T, in a novel genus with the orthographically incorrect name 'Labrys monachus' (Vasil’eva & Semenov, 1984). The name of this strain has been validly published as Labrys monachus (Vasil’eva & Semenov, 1985). Cells of this strain consisted of flat, triangular cells with prosthecae in two of the three corners, which allows clear morphological distinction from other genera of budding bacteria. So far, the phylogenetic position of L. monachus VKM B-1479T has not been determined.

The first ‘mushroom-shaped’, budding bacterium was described by Whittenbury & Nicoll (1971). The authors isolated strain NCIMB 1785T from fresh pond water; this strain differed from previously described budding bacteria in a number of properties, including dividing mode, cell morphology and fine structure (Whittenbury & Nicoll, 1971). This strain has not yet been assigned to a genus or species. A morphologically similar, non-motile, Gram-negative strain (Z-2821 = VKM B-1335T = DSM 5895T) was isolated in 1972 from a cumulative culture of methane-oxidizing bacteria that were sampled from a lowland marsh in Abramtsevo, near Moscow (Namsaraev & Zavarzin, 1973, 1974). Vasil’eva and co-workers (Lafitskaya & Vasil’eva, 1976; Vasil’eva et al., 1980) reported an additional mushroom-shaped bacterial strain, Z-1109 (=VKM B-1336), and proposed a novel genus, Angulomicrobium, with the type species Angulomicrobium tetraedrale VKM B-1335T (Vasil’eva et al., 1986). Despite certain morphological and physiological dissimilarities to strain VKM B-1335T, these authors included strain VKM B-1336 in the genus Angulomicrobium without attributing it to a particular species (Vasil’eva et al., 1980). Two additional strains that resembled ‘mushroom-shaped bacteria’ were isolated by Stanley et al. (1976). So far, no data on the taxonomic affiliation of ‘mushroom-shaped bacteria’ have been published.

The aim of the present work was to determine the taxonomy of the type strains of the genera Labrys, Stella and Angulomicrobium by lipid analysis and comparison of 16S rRNA gene sequences. Furthermore, the phylogenetic positions of strains NCIMB 1785T and VKM B-1336 have been determined, resulting in the proposal of Angulomicrobium anamnifoand sp. nov., with the type strain NCIMB 1785T.

**METHODS**

**Strains.** The strains used in this study and their origins are listed in Table 1. All strains were grown in freshwater Caulobacter medium PYEM [2 g peptone, 2 g yeast extract, 0.5 g NH4Cl, 1 l MilliQ water (Millipore)]. After autoclaving and cooling, 5 ml sterile-filtered riboflavin (0.2 mg ml⁻¹), 2 ml 50% sterile glucose, 1 ml 20% sterile MgSO4 and 1 ml 10% sterile CaCl2 were added. For lipid analysis, strains were grown in 2 l flasks at 30 °C and 100 r.p.m. and biomass was harvested in the late-exponential phase after 72 h. Due to their very slow growth, Stella strains were harvested after 2 weeks.

**Spectroscopic DNA–DNA hybridization.** DNA was isolated by chromatography on hydroxyapatite, according to the procedure of Cashion et al. (1977). DNA–DNA hybridization in 2 x SSC + 10% (v/v) DMSO at 69 °C was carried out as described by De Ley (1967) with the modification described by Huss et al. (1983) and Escara & Hutton (1980), using a model 2600 spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford Instrument Laboratories). Renaturation rates were computed with the program TRANSFER.BAS (Jahnke, 1992).

**16S rDNA sequencing.** Almost-complete 16S rRNA genes were amplified by PCR from the strains listed in Table 1 and were sequenced as described previously (Abraham et al., 1999). Resulting sequences were aligned with reference 16S rRNA gene sequences (Stoesser et al., 2002; Cole et al., 2003) by the ARB program package (Ludwig et al., 2003), using the evolutionarily conserved primary sequence and secondary structure as references (Gutell et al., 1985). Evolutionary distances (Jukes & Cantor, 1969) were calculated from pairwise similarities of complete sequences by using only homologous, unambiguously determined nucleotide positions. A phylogenetic tree was constructed by using the DNADIST and FITCH programs of the PHYLIP package (Felsenstein, 1989).

**Lipid analysis**

**Polar lipid fatty acid analysis.** Lipids were extracted by using a modified Bligh–Dyer procedure (Bligh & Dyer, 1959) and fatty acid methyl esters were generated and analysed by GC, as described previously (Vancanneyt et al., 1996).

**Table 1.** Strains used in this study and their origin

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
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<tr>
<td><strong>Angulomicrobium:</strong></td>
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<tr>
<td>Angulomicrobium tetraedrale DSM 5895T</td>
<td>Lowland bog near Moscow, Russia</td>
</tr>
<tr>
<td>Angulomicrobium tetraedrale VKM B-1335T</td>
<td>Lowland bog near Moscow, Russia</td>
</tr>
<tr>
<td>[Angulomicrobium] VKM B-1336</td>
<td>Peat bog soil near Moscow, Russia</td>
</tr>
<tr>
<td>Mushroom-like bacteria NCIMB 1785T</td>
<td>Fresh pond water</td>
</tr>
<tr>
<td>Labrys monachus VKM B-1479T</td>
<td>Lake Mustijaër, Estonia</td>
</tr>
<tr>
<td><strong>Stella:</strong></td>
<td></td>
</tr>
<tr>
<td>Stella humosa DSM 5900T</td>
<td>Virgin chernozem, A1 layer, Krasnodar region, Russia</td>
</tr>
<tr>
<td>Stella vacuolata DSM 5901T</td>
<td>Sewage sludge from pig farm, St Petersburg region, Russia</td>
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</tbody>
</table>
Tandem mass spectrometry (MS). Fast atom bombardment-MS in the negative mode was performed on the first of two mass spectrometers of a tandem, high-resolution instrument (JMS-HX/HX110A; JEOL) as described previously (Abraham et al., 1997). A mixture of triethanolamine and tetramethylurea was used as the matrix. Negative daughter ion spectra were recorded by using all four sectors of the tandem mass spectrometer. Helium served as the collision gas for generating collision-induced dissociation (CID) for identification of prominent molecular ions.

RESULTS AND DISCUSSION

16S rRNA gene sequence analysis

As revealed by 16S rRNA gene sequence comparison, the strains of Angulomicrobium, Labrys and Stella that were studied were found to cluster with the ‘Alphaproteobacteria’ (Fig. 1). Within this subclass, the strains formed distinct branches that appeared to be well-separated from other bacterial genera. Despite their common radial symmetry and, with the exception of Angulomicrobium strains, the presence of prosthecae, these genera displayed no obvious closer relationships. This corroborates with the finding of the polyphyletic origin of stalks in caulobacteria (Stahl et al., 1992; Abraham et al., 1999).

Genus Stella

Both type strains of Stella species, DSM 5900T and DSM 5901T, were found to form a well-separated monophyletic group (Fig. 1) that showed only distant relationships to published 16S rDNA sequences of type strains of other bacterial genera. The 16S rRNA gene sequences from genera with validly published names that were most similar to those of the Stella cluster were from strains that belonged to the genera Azospirillum, Magnetospirillum and Aquaspirillum, with sequence similarities that ranged between 87 and 90 %. This placed the genus Stella in the order Rhodospirillales in the family Rhodospirillaceae and not in the Acetobacteraceae, as has been assumed previously (Garrity et al., 2001). Comparison of the 16S rRNA gene sequences revealed 99-0 % similarity between S. humosa DSM 5900T and S. vacuolata DSM 5901T. This close 16S rRNA gene sequence similarity is not reflected by data from DNA–DNA hybridization experiments; Reimer & Schlesner (1989) determined a DNA–DNA similarity as low as 15 % between the type strain of S. humosa and S. vacuolata.
strains of *S. humosa* and *S. vaculata*, thus pointing to substantial differences in the genetic structure of these strains.

Contrary to DNA–DNA reassociation data, the genus *Stella* appears to be quite consistent in terms of morphology, physiology, nutrient requirements and DNA G+C content (approx. 67–73 mol%) (Hirsch & Schlesner, 1981; Vasilyeva, 1985). Major distinguishing traits are the occurrence of gas vacuoles in some strains and low DNA–DNA hybridization values that are reported between a number of strains.

Mass spectra of the phospholipid fractions from *Stella* spp. showed mainly molecular ions with even mass numbers, pointing to the presence of mainly nitrogen-bearing phospholipids. Although CID-MS for identification of compounds could not be run, due to scarcity of material, the recorded masses, together with the fatty acids found in the polar lipid fraction, strongly favour the presence of phosphatidylethanolamine and phosphatidylethanolamine. Additionally, two unidentified lipids with molecular ions at *m/z* 893 and 1042 were observed. Sittig & Schlesner (1993) reported the presence of phosphatidyl N-methyl ethanolamine, phosphatidylcholine and cardiolipin for *Stella* strains; however, the latter could not be detected in the mass spectra. Furthermore, they found relatively high amounts of long-chain hydroxy fatty acids.

**Genus Labrys**

*L. monachus* VKM V-1479T exhibited 16S rRNA gene sequence similarities of 91–92% to the most closely related genera, namely species of *Mesorhizobium*, *Rhodopseudomonas* and *Azorhizobium* (Fig. 1). This confirmed the separation of *L. monachus* VKM B-1479T at the genus level, as has been suggested previously on the basis of morphological and physiological data (Vasil’eva & Semenov, 1984). 16S rRNA gene sequence data did not allow us to affiliate *B. ploughmanii* with any of the most closely related families that were suggested by Garrity et al. (2001), namely *Rhizobiaceae*, *Brucellaceae*, *Phyllobacteriaceae*, *Methyloxydaceae*, *Beijerinckiaceae* and *Bradyrhizobiaceae*. Additionally, *L. monachus* VKM B-1479T is not affiliated to the family *Hyphomicrobiaceae*, as has been suggested by Garrity et al. (2001). VKM B-1479T may be assigned to a separate family, ‘Labryaceae’, in the future. However, due to the availability of only one strain for characterization, it is too early to resolve the affiliation of *L. monachus* VKM B-1479T at family level.

Four different types of polar lipid could be detected in this strain: phosphatidylglycerol, phosphatidyl N,N-dimethylethanolamine, phosphatidylcholine and a lipid with a mass of 827 Da that belonged to an unknown type of phospholipid. With the aid of CID-MS, most compounds were elucidated. CID of the (M–H)+ ion yielded abundant carbonate anions from both the sn-1 and the sn-2 position, thus allowing identification of the fatty acids attached to the different lipids. In addition, there were neutral losses of the sn-2 and sn-1 substituent as free carboxylic acid, as well as loss of each fatty acyl group as a substituted ketone. Furthermore, the positions of the fatty acids at the glycerol backbone could be determined. For the fatty acid positioned at sn-2, neutral loss as free fatty acid, as well as substituted ketene, is more frequent than for that at sn-1 (Murphy & Harrison, 1994). By this method, the structure of the phospholipids was identified (Abraham et al., 1997); Table 2 summarizes the results. Several differences in the structure of the phospholipids can be found between *L. monachus* VKM B-1479T and *A. tetradrae* DSM 5895T (Table 2), which can be used to differentiate between these two genera. Such differentiation was not possible on the basis of phospholipid types alone (Sittig & Schlesner, 1993).

Fatty acids found in the polar lipid fractions of *L. monachus* VKM B-1479T, *A. tetraedrae* DSM 5895T and strain NCIMB 1785T are listed in Table 3, together with the fatty acids of the whole-cell hydrolysate of strain NCIMB 1785T. The fatty acids of *L. monachus* VKM B-1479T were similar to those of *Angulomicrobium* strains, but the amount of C18:1ω7 was rather low, whilst that of C19:0ω8:9 was the highest of all strains in this study, confirming an earlier report by Sittig & Schlesner (1993).

**Genus Angulomicrobium**

The 16S rRNA gene sequences of *A. tetraedrae* VKM B-1335T and DSM 5895T were identical. 16S rRNA gene analysis of *A. tetraedrae* VKM B-1335T (= DSM 5895T) confirmed the status of this taxon as a distinct genus and species and the placement of this genus in the family *Hyphomicrobiaceae*. The closest cultivated relatives of *A. tetraedrae* as deduced by 16S rRNA gene sequencing were *Methylorhabdus multi- vorans*, *Starkeya novella* and *Ancylobacter aquaticus*, with sequence similarities that ranged between 96 and 97%. Additionally, high sequence similarities (> 96%) to molecular clones of thiosulfate-oxidizers from rice fields (Stubner et al., 1998) and to *Xanthobacter* spp. were detected. However, several traits permit distinction between members of the genus *Angulomicrobium* and their close relatives. *Angulomicrobium* differs from *Starkeya* in terms of cell morphology and replication, lack of a chemolithotrophic nutrition mode and utilization of the Entner–Doudoroff pathway for breakdown of sugars by the former (Vasil’eva et al., 1980; Kelly et al., 2000). Furthermore, *Angulomicrobium* can be distinguished from *Methylorhabdus* by its budding mode of replication, presence of oxidase activity, inability to reduce nitrate and by lower amounts of C16:0, but higher amounts of C14:0 and C18:1ω7 in its cellular fatty acids (Vasil’eva et al., 1980; Doronina et al., 1995).

Strain VKM B-1336, which was originally proposed to be a member of the genus *Angulomicrobium*, was included in the genus description (Vasil’eva et al., 1980) despite observed differences in morphology, substrate utilization, ultrastructure and mode of division. We determined overall 16S rRNA gene sequence similarity between strains VKM B-1336 and VKM B-1335T (= DSM 5895T) to be as low as 91%. This points to a remote relationship above genus level between
Strain NCIMB 1785T shared 99·4 % 16S rDNA sequence similarity with strain DSM 5895T (= DSM 5895T). This confirmed the affiliation of strain NCIMB 1785T with the genus *Angulomicrobium*, which was suggested by earlier morphological and physiological data (Whittenbury & Nicoll, 1971; Stanley et al., 1976; Vasil’eva et al., 1980). DNA–DNA hybridization between *A. tetraedrale* DSM 5895T and strain NCIMB 1785T gave 60–6·6 % DNA–DNA similarity. An accepted recommendation by Wayne et al. (1987) suggested that strains that share > 70 % DNA–DNA similarity should be included in the same species. Therefore, we propose that strain NCIMB 1785T should be placed in a novel species, *Angulomicrobium amanitiforme* sp. nov.

Four different types of polar lipids were detected in strains DSM 5895T and NCIMB 1785T: phosphatidylglycerol, phosphatidyl N,N-dimethyllethylamine, phosphatidylcholine and lipids that belonged to an unknown type of phospholipid (Table 2). Phosphatidylglycerol was only found in *A. tetraedrale* DSM 5895T; strain NCIMB 1785T lacked this class of phospholipids. From the structure of the different phospholipids, it is apparent that strain NCIMB 1785T preferably more than strain DSM 5895T, and that all main lipids of strain NCIMB 1785T had this fatty acid. *Angulomicrobium* strains were the only strains in this study to possess unidentified lipids with masses of 932 and 946 Da. Their CID spectra all showed the formation of glycerophosphatic acid (GPA) ions, identifying this group of polar lipids as phospholipids. Analysis of GPAs revealed the fatty acids for which the amount for all taxa was > 1 % are not given. TR, Trace amount (< 1 %); –, not detected.

In this study, it was demonstrated for the two type strains of *Stella* species that high 16S rDNA sequence similarity (≥ 99 %) does not necessarily correspond to high DNA similarity [DNA–DNA hybridization value < 15 %; data from Reimer & Schlesner (1989)]. This well-known phenomenon (Regenhardt et al., 2002; Stackebrandt et al., 2002 and references therein) also applies to the genus *Angulomicrobium*. Although strains NCIMB 1785T and VKM B-1335T share 99·4 % 16S rRNA gene sequence similarity, they table...
are not related at species level, as shown by DNA–DNA hybridization. Aside from this, strain NCIMB 1785T differs from A. tetraedrale VKM B-1335T by the use of citrate, D-(−)-ribose and L-serine as substrates and the inability to utilize D-(+) -malate, D-(+) -mannose, D-(+) -melibiose, methylamine hydrochloride and L-(+) and D-(−)-tartrate as substrates.

**Description of Angulomicrobium amanitiforme sp. nov.**

*Angulomicrobium amanitiforme* (a.m.ni’ti.for.me. N.L. n. Amanita name of fungal genus; L. adj. suffix -formis -is-e-like, of the shape of; N.L. neut. adj. amanitiforme formed like a toadstool).

The description of the species is as that for the genus, with the following additions. Cells are non-motile, non-spore-forming, capsulated and irregularly shaped. They are 1·0 × 1·5 μm in size with radial symmetry and show tetrahedral cell morphology during the early stages of cell replication. A complex membranous system is not developed, but in addition to the cytoplasmic membrane, some peripherally aligned membranes and a membranous cell body at the site of cell division are present. The budding process is initiated by doubling the tube part of the cell, followed by enlargement of the tube part end-section. Finally, symmetric fission of the cell occurs with smaller, non-slimy colonies. Colonies are white, round and mucous with a pearly shine. However, there is a second colony morphology with smaller, non-slimy colonies. Nutritional type is chemoorganotrophic. CO₂ in the presence of H₂ is not utilized as a sole carbon and energy source. Organic growth factors are not required, but do stimulate growth. Glucose, arabinose, galactose, fructose, mannitol, glycerol, formate, acetate, propionate, lactate, fumarate, succinate, citrate and glutarate are used as sole carbon and energy sources, whereas mannose, lactose, malate, succrose, dulcitol, butyrate, urea, glycine and methanol are not. Contrary to VKM B-1335T, strain NCIMB 1785T utilizes citrate, D-(−)-ribose and L-serine and does not utilize D-(+)-malate, D-(+)-mannose, D-(+)-melibiose, methylamine hydrochloride or L-(+)- or D-(−)-tartrate. Metabolism is strictly aerobic. Catalase and oxidase activities are present. Nitrate, carbonate and sulfate are not utilized as electron acceptors. Cytochromes a, b and c are present. Acids are produced from sugar and sugar alcohols. Growth temperature ranges from 15 to 40 ℃, with an optimum growth temperature between 28 and 30 ℃. At 30 ℃, growth occurs at pH values between 5·2 and 8·0, with an optimum at pH 6·8-7·0. Main phospholipids are phosphatidyl N,N-dimethylethylamine and phosphatidylcholine, containing at least one C₁₉₋₂₀:0,8,9 cyclopentyl fatty acid. DNA G+C content is 67·7 mol% for the type strain. 16S rRNA gene sequencing places this species in the ‘Alpha-proteobacteria’. DNA–DNA hybridization between the type strain and *A. tetraedrale* DSM 5895T was 60·6%.

The type strain of the species is NCIMB 1785T (= DSM 15561T).

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