Phylogenetic analysis of some *Sporomusa* sub-branch members isolated from human clinical specimens: description of *Megasphaera micronuciformis* sp. nov.

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Two unknown, Gram-negative, catalase-negative and strictly anaerobic cocci were isolated from two independent human samples (strains AIP 49.01 and AIP 412.00T). Comparative 16S rRNA gene sequencing demonstrated that these two organisms displayed 99.8% sequence identity and that they are members of the *Sporomusa* sub-branch of the low-G+C Gram-positive bacteria. The most closely related 16S rDNA sequences were from *Megasphaera* sp. oral clone BU057 (99.8%) and from isolates of *Megasphaera cerevisiae* and *Megasphaera elsdenii* (94.5% and 93.8%, respectively). Phylogenetic analysis based on 16S rDNA sequences showed that these two strains were most closely related to *M. elsdenii* and belonged to the *Megasphaera* genus. Differences from previously described *Megasphaera* species in terms of size, biochemical tests (particularly the analysis of metabolic end products), gas production and DNA G+C content indicated that the two strains studied represent a novel species of anaerobic Gram-negative cocci. The name *Megasphaera micronuciformis* sp. nov. is proposed for these two isolates. It is also proposed that the uncultured organism previously deposited as *Megasphaera* sp. oral clone BU057 should be named ‘*Candidatus Megasphaera micronuciformis*’. The type strain of *Megasphaera micronuciformis* is AIP 412.00T (= CIP 107280T = CCUG 45952T).

In phenetic taxonomy, all the strictly anaerobic Gram-negative cocci were originally classed as a single family, the *Veillonellaceae* (Rogosa, 1971, 1984). Three genera were accepted into this family: *Veillonella*, *Acidaminococcus* and *Megasphaera*. Recently, *Anaeroglobus* gernitus gen. nov., sp. nov. was described (Carlier et al., 2002). This genus, consisting of strictly anaerobic Gram-negative cocci isolated from human samples, was included in the family *Veillonellaceae*. Phylogenetic taxonomy showed that the four previous genera, despite the negative Gram stain, belonged to the *Sporomusa* sub-branch of the *Firmicutes* (Carlier et al., 2002). More recently, in Bergey’s *Manual of Systematic Bacteriology* (Garrity & Holt, 2001) all members of the *Sporomusa* sub-branch were grouped in the family *Acidaminococcaceae*. As a consequence, the family *Veillonellaceae* was excluded from this new classification.

In recent studies, culture-independent methods based on 16S rDNA cloning and sequence analysis have been used to determine the bacterial diversity of oral flora (Paster et al., 2001; Rolph et al., 2001). Consequently, an increasing number of sequences corresponding to uncultivated bacteria belonging to the *Sporomusa* sub-branch have been deposited in databases. These results suggest that many members of this phylogenetic branch remain unknown. Furthermore, in a routine medical microbiology exercise, we isolated five unidentified strains affiliated to the *Sporomusa* sub-branch on the basis of 16S rDNA sequences. Although three of them are not completely characterized, they seem...
to be new representatives of the genera Dialister, Veillonella and Acidaminococcus (strains ADV 04.01, ADV 281.99 and ADV 255.99, respectively). Thus, we have included their 16S rDNA sequences in our phylogenetic study. The two other strains (strains AIP 49.01 and AIP 412.00$^\text{T}$) were analysed further. These anaerobic coccoid organisms exhibited a Gram-negative stain and could not be assigned to any defined taxon on the basis of classical phenotypic criteria. Phenotypic and phylogenetic analysis of these two clinical isolates allowed us to propose a new species belonging to the genus Megasphaera. Furthermore, a general phylogenetic analysis of the Sporomusa sub-branch was performed.

**Bacterial strains and isolation**

Strains AIP 49.01 and AIP 412.00$^\text{T}$ were recovered from clinical samples obtained from two patients admitted to the University Hospital of Montpellier. These two strains were both recovered from mixed aerobic–anaerobic flora. The first strain, AIP 412.00$^\text{T}$, was isolated in September 2000 from a liver abscess in a 32-year-old woman hospitalized in a medical unit. The second strain, AIP 49.01, was isolated in January 2001 from a pus sample from a 71-year-old woman admitted to the emergency unit for a whitlow.

These two samples were incubated on Columbia sheep blood agar for 4 days in an anaerobic jar with AnaeroGen System (Oxoid); this yielded mixed aerobic–anaerobic cultures. Strain AIP 412.00$^\text{T}$ was cultured with *Escherichia coli*, coagulase-negative *Staphylococcus* sp. and pigmented *Prevotella* sp., and strain AIP 49.01 was cultured with *Haemophilus parainfluenzae*, *Enterococcus faecalis*, *Klebsiella oxytoca*, *Prevotella* sp. and *Peptostreptococcus* sp.

**Colony and cell morphology**

Colonies of both strains appeared on blood agar after 2–3 days incubation. The colonies were circular, convex, shiny, translucent with a smooth surface and approximately 0.5–1.0 mm in diameter, non-pigmented and non-haemolytic. Strains AIP 49.01 and AIP 412.00$^\text{T}$ are cocci, occur as single cells and display a Gram-negative stain. They are strictly anaerobic and non-motile, and endospores are not formed. For negative staining, bacterial suspensions were dispersed in 0.05% ammonium acetate. A 200-mesh copper grid, coated with parlodion (Electron Microscopy Sciences) and reinforced with a thin coat of carbon, was held on the bacterial suspensions and left for 20 min. After washing with a drop of 0.05% acetic acid ammonium for 1 min, grids were negatively stained with uranyl acetate for 1 min. Samples were observed under a Hitachi H7100 electron microscope (Fig. 1a). Cocci varied in diameter from 0.4 to 0.6 μm, and cells showed a convoluted surface. For electron microscopy (EM) of ultrathin sections, bacteria were fixed in 2.5% glutaraldehyde in Milloning’s buffer (0.1 M, pH 7.3) for 1 h at 4 °C. Post-fixation, the grids were treated with 2% osmium tetroxide (Electron Microscopy Sciences) for 1 h, followed by treatment with 0.5% tannic acid for 30 min. The specimens were dehydrated through an ethanol series and embedded in Embed 812 (Electron Microscopy Sciences). Ultrathin sections were cut with a Reichert OMU2 ultramicrotome and picked up with 300-mesh copper grids. Sections were stained with lead citrate and uranyl acetate, using the procedure of Reynolds (1963). Observation at low magnification confirmed the overall morphology observed after negative staining (Fig. 1b). At higher magnification, three separate structural entities were observed in the cell wall, and will be referred to as the outer membrane, the peptidoglycan layer and the cytoplasmic membrane (Fig. 1c).

**Biochemical characteristics**

Biochemical reactions were performed according to the procedures described by Holdeman et al. (1977). Metabolic end products were analysed by quantitative GC as described previously (Carlier, 1985). An API Rapid ID 32A kit (bioMérieux) was used for enzymic profile determination, as recommended by the manufacturer. These

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**Fig. 1.** Ultrastructure of strains AIP 49.01 and AIP 412.00$^\text{T}$: (a) general morphology after negative staining; (b) general morphology after EM of ultrathin sections; (c) cell wall and membranes after EM of ultrathin sections. OM, outer membrane; MU, peptidoglycan layer; CM, cytoplasmic membrane. Bars, (a) 178 nm; (b) 333 nm; (c) 66 nm.
strictly anaerobic cocci were unreactive in most of the conventional biochemical tests. No gas was produced by glucose agar deep cultures. Catalase activity and indole production were not detected. Gelatin was not liquefied and milk was not modified. Nitrate reduction was negative and lactate was not fermented. No pigment was observed on laked sheep blood agar. Desulfoviridin was produced by strain AIP 412.00T. By using presumptive identification tests, both strains were susceptible to 5 μg vancomycin, 1 mg kanamycin, 10 μg colistin, 4 μg metronidazole and bile discs. Acid was not produced from aesculin, arabinose, cellobiose, fructose, galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, starch, sucrose, trehalose or xylose. Aesculin was not hydrolysed. The major metabolic end products (mM) for strains AIP 412.00T and 49.01 were (respectively): acetic acid (3-0, 3-6), propionic acid (2-2, 1-7), isobutyric acid (0-0, 0-3), butyric acid (2-1, 2-1), isovaleric acid (3-2, 2-3), valeric acid (0-0, 2-4) and 2-phenylacetic acid (0-8, 0-9). Lactic and succinic acids were not produced. The type strain AIP 412.00T and reference strain AIP 49.01 yielded negative reactions in all Rapid ID 32A tests. Tests that serve to distinguish these new cocci from other closely related anaerobic Gram-negative bacteria are given in Table 1.

DNA G+C content, 16S rDNA sequence analysis and genomic organization

The DNA G+C content of strain AIP 412.00T was 46.4 mol%, determined by HPLC at the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). For 16S rDNA amplification by PCR, DNA was rapidly extracted from a single colony by a boiling/freezing method (Woods et al., 1993). The crude extracts (2 μl) were used as the template for PCR experiments. 16S rDNA was selectively amplified using the primers 5’-GTGCTGCAGAGATTTGATCGCTGCAG-3’ (270) and 5’-CACGGATCCAGGATGTCGACAGTTT (1492r) as described previously (Carlier et al., 2002). The PCR product (about 1-4 kb) was directly sequenced in both directions by Genome Express (France) on an Applied Biosystems automatic sequencer; almost-complete 16S rDNA sequences (1416 bp) were determined for strains AIP 49.01 and AIP 412.00T. The alignment of the two sequences revealed 99.8 % similarity between them. The comparison with known sequences in the database using the BLAST program (Altschul et al., 1997) showed that the sequences were most similar to those of members of the Sporomusa sub-branch of the class Clostridia of the phylum Firmicutes, corresponding to cluster IX as described by Collins et al. (1994). Alignment of the sequences showed that strains AIP 49.01 and AIP 412.00T are most closely related to the species Megasphaera elsdenii, Megasphaera cerevisiae, A. geminatus and Dialister pneumosintes, with sequence identities of 93-8, 94-5, 93-8 and 89-2 %, respectively. However, the highest identity (99-8 %) was observed with the sequence of the uncultured bacterium Megasphaera sp. oral clone BU057.

Recently, large-scale chromosome structure has been described as a sensitive indicator of phylogenetic relationships between bacteria (Liu et al., 1999). Mapping experiments with the restriction enzyme I-CeuI were undertaken to measure the bacterial chromosomes and to determine the rrs skeletons of strain AIP 49.01, strain AIP 412.00T and M. elsdenii. Intact DNA, prepared in agarose plugs as previously described (Marchandin et al., 2001), was digested by I-CeuI (New England Biolabs) according to the manufacturer’s recommendations. I-CeuI fragments

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<th>Table 1. Characteristics useful for differentiating species belonging to the genera Megasphaera and Anaeroglobus</th>
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<td>Taxa (reference): 1, <em>Megasphaera micronuciformis</em> (this study); 2, <em>Megasphaera elsdenii</em> (Rogosa, 1984); 3, <em>Megasphaera cerevisiae</em> (Engelmann &amp; Weiss, 1985); 4, <em>Anaeroglobus geminatus</em> (Carlier et al., 2002). +, Positive; −, negative; +/−, variable.</td>
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<td>Characteristic</td>
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*A, acetic acid; P, propionic acid; iB, isobutyric acid; B, butyric acid; iV, isovaleric acid; V, valeric acid; C, caproic acid; PhA, 2-phenylacetic acid. Parentheses indicate an inconstant production.*
were separated by PFGE using pulse ramps of 90–150 s in a CHEF-DRII apparatus (Bio-Rad) on 0·8 % agarose gel in 0·5× TBE (data not shown). The chromosomes of strains AIP 49.01 and AIP 412.00 were 1780 and 1837 kb in size, respectively. A difference of 3·1 % in chromosome size was commonly observed between strains of the same species (Berghorsonn & Ochman, 1998). I-Cell profiles for the two strains showed that they both possess four rrn operons in the same locations on the chromosome. In contrast, the chromosome of M. elsdenii was 2578 kb in size, and carried seven rrn copies. The equivalent genome size and identical rrn skeleton, together with the high percentage identity in 16S rDNA sequence and phenotypic data, suggested that strains AIP 49.01 and AIP 412.00 belonged to the same species.

16S rDNA-based phylogeny of the Sporomusa sub-branch and the genus Megasphaera

Forty-one 16S rDNA sequences were included in the analysis of the Sporomusa sub-branch. Thirty-eight of these corresponded to reference sequences related to strains AIP 49.01 and AIP 412.00, together with representative sequences from Sporomusa sub-branch clusters and lineages. Three other 16S rDNA sequences, obtained from unidentified clinical strains isolated in our laboratory (strains ADV 04.01, ADV 281.99 and ADV 255.99) were included, since BLAST analysis showed that they also belonged to the Sporomusa sub-branch. An analysis focused on the genus Megasphaera was also performed. Twenty 16S rDNA sequences from all strains and DNA clones representing the genera Megasphaera and Anaeroglobus, together with strains AIP 49.01 and AIP 412.00, were included in the analysis. Dialisr pneumosintes was included as an outgroup.

The sequences were aligned using the program DIALIGN (Morgenstern, 2002) for proper analysis of the evolutionary relationships of the strains studied in this work. This alignment was then checked manually to remove gaps and ambiguously aligned positions. The phylogenetic analyses were performed with the PHYLIP program package (Felsenstein, 1993). Pairwise evolutionary distances were computed using the Jukes–Cantor equation implemented in the DNADIST program, and phylogenetic trees were constructed by neighbour-joining with a random input order of sequences. The significance of the nodes was tested by bootstrap analysis to produce 1000 trees, then a consensus tree was compiled using the CONSENSE program. Maximum-likelihood and maximum-parsimony analyses of sequence data and least-squares analysis of evolutionary distances were also performed, using the programs DNAML, DNPARS and FITCH, respectively. Whatever the method used to reconstruct the trees, they exhibited an identical overall structure. A representative evolutionary distance tree of members of the genera Megasphaera and Anaeroglobus, computed using the Jukes–Cantor equation and neighbour-joining, is shown in Fig. 2. The neighbour-joining tree comprising 41 sequences, representative of the entire Sporomusa sub-branch, is available as supplementary data in IJSEM Online (http://ijis.sgjmjournals.org).

In the larger tree, strains AIP 49.01 and AIP 412.00 branched together with the Dialisr–Megasphaera–Anaeroglobus group and formed a separate subgroup from the genus Veillonella, by a robust node with a bootstrap value of 98 %. In this subgroup, M. elsdenii, strain AIP 49.01, strain AIP 412.00 and Megasphaera sp. oral clone BU057 grouped together, whereas M. cerevisiae and A. geminisus individually were more deeply branched. Moreover, D. pneumosintes formed a separate lineage. Consequently, D. pneumosintes was used as the outgroup to construct the tree focused on the genera Megasphaera and Anaeroglobus (Fig. 2). In opposition to the branching order observed on the previous tree, strains AIP 49.01, AIP 412.00 and clone BU057 grouped together and then grouped with M. cerevisiae. M. elsdenii species and three uncultured bacteria formed a large homogeneous group. The 16S rDNA sequence of the uncultured bacterium HuCB85 seemed to form a separate lineage from M. elsdenii. The group formed by A. geminisus and related clones was more deeply branched. This discordance between the two trees was further shown by low bootstrap values at the corresponding nodes, and also appeared by comparison with a previously published phylogram (Carlier et al., 2002). Thus, an absolute branching order in the Megasphaera–Anaeroglobus group remains uncertain.

The 16S rDNA sequence similarity and phylogeny suggested that strains AIP 49.01 and AIP 412.00 belong to the genus Megasphaera. Since the morphology, metabolic end products, DNA G+C content, 16S rDNA sequence and genomic organization of strains AIP 49.01 and AIP 412.00 are distinct from those of the previously described Megasphaera species, they appear to belong to a novel species within this genus. We propose to assign the name Megasphaera micronuciformis to strains AIP 49.01 and AIP 412.00. We also suggest that the sequence deposited as Megasphaera sp. oral clone BU057 has been obtained from a third member of the novel species, Megasphaera micronuciformis sp. nov. Thus, this uncultivated strain should be named ‘Candidatus Megasphaera micronuciformis’ strain BU057 (Murray & Schleifer, 1994).

Strains AIP 49.01 and AIP 412.00T have been included in a novel species of the genus Megasphaera, rather than creating a new genus, because of two main arguments: (i) creating a new genus would split M. elsdenii and M. cerevisiae into a paraphyletic genus; (ii) phylogenetic data indicated uncertainty in the branching order between strains AIP 49.01, AIP 412.00, A. geminisus, M. elsdenii and M. cerevisiae. Thus, it appeared to us that it was more cautious to include the two strains in the existing genus Megasphaera. Further investigations, particularly chemotaxonomic analysis and phylogeny based on markers other than 16S rDNA, may enable a better delineation and reappraisal of taxa that belong to the Megasphaera–Anaeroglobus group.
At a phylogenetic level, strains of the *Sporomusa* sub-branch were shown to belong to the Gram-positive phylum. The Gram-negative staining of *Megasphaera* spp. was not an artefact as previously suggested (Gupta, 1998); EM observations of the outer membrane showed that the bacterial cell wall was indeed of the Gram-negative type, as previously described for *Veillonella* spp. (Bladen & Mergenhagen, 1964), *Selenomonas ruminantium* (Kamio & Takahashi, 1980; Kalmokoff et al., 2000) and *Centipeda periodontii* (Males et al., 1984). The type of peptidoglycan is considered to be a reliable criterion for bacterial classification, as the dichotomy of Gram-positive and Gram-negative species was confirmed by molecular phylogenies based on 16S rRNA and HSP70 gene sequences (Gupta, 1998). The *Sporomusa* sub-branch is an exception to this general rule that correlates the cell-wall structure with the phylogenetic placement of a bacterium.

Bacterial diversity among the *Sporomusa* sub-branch seems to be largely underestimated, as suggested by the number of 16S rRNA gene sequences deposited for unidentified oral clones in previous studies (Paster et al., 2001; Rolph et al., 2001). Moreover, we have found three bacterial strains (*Dialister* sp. clinical strain ADV 04.01, *Veillonella* sp. clinical strain ADV 281.99 and *Acidaminococcus* sp. clinical strain ADV 255.99) that are not yet completely characterized, and may represent novel taxa within the *Sporomusa* sub-branch. Indeed, the 16S rRNA gene sequences determined for these clinical isolates showed less than 97% sequence identity with the most closely related species (92-2% with *Dialister pneumosintes* ATCC 33048T, 94-5% with *Veillonella atypica* DSM 20739T and 95-5% with *Acidaminococcus fermentans* DSM 20731T).

The phylogenetic analysis extended to the overall *Sporomusa* sub-branch showed that the family *Veillonellaceae*, previously described by cocccoid morphology, Gram-negative staining and phenotype, did not represent a true clade. Thus, the genus *Acidaminococcus* was very distant from the genera *Veillonella* and *Megasphaera*, whereas the genus *Dialister* (containing Gram-negative anaerobic bacilli) branched with these two genera. In a polyphasic approach including phylogeny, the family *Veillonellaceae* could not be considered as a relevant taxon. Although the family *Veillonellaceae* remains in the amended edition of the Approved Lists of Bacterial Names (Skerman et al., 1989), this family has been suppressed from the classification proposed in the latest edition of Bergey’s *Manual of Systematic Bacteriology* (Garrity & Holt, 2001). This classification grouped all genera belonging to the *Sporomusa* sub-branch.
in a single large family named ‘Acidaminococccae’. The phylogenetic analysis of the Sporomusa sub-branch is in accordance with the proposal of this new classification.

Emendation of the description of the genus Megasphaera

Cocci, 0·4–2·0 μm or more in diameter. Gas may or may not be produced. Lactate and fructose may or may not be fermented. Found in the rumen of cattle and sheep, in the faeces and intestine of man, in human clinical specimens and in spoiled bottled beer. The DNA G+C content is 42·4–46·4 mol% (Tm) and 53·6 mol% (Bd).

Description of Megasphaera micronuciformis sp. nov.

Megasphaera micronuciformis (mic.ro.nu.ci.for’mis. Gr. adj. micros small; L. fem. gen. n. nuci of a nut; L. fem. adj. formis shape or form; N.L. fem. adj. micronuciformis small walnut-shaped, referring to the morphology of bacterial cells and cell surface).

Cells are Gram-negative, coccoid, usually single, 0·4–0·6 μm in diameter, non-motile. Endospores are not formed. Strictly anaerobic. These bacteria are non-fermentative and non-proteolytic (gelatin- and milk-negative). Nitrate is not reduced and indole and catalase are not produced. Desulfoviridin may be produced. Metabolic end products are acetic, propionic, butyric, isovaleric and 2-phenylacetic acids. Valeric acid and trace amounts of isobutyric acid may be produced. Genomic DNA G+C content is 46·4 mol%. Differentiated from other Megasphaera species by size, DNA G+C content, metabolic end products and 16S rDNA gene sequence.

The type strain is AIP 412.00T (= CIP 107280T = CCUG 45952T) and the reference strain is AIP 49.01. Habitat is unknown.

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References


