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Mucispirillum schaedleri gen. nov., sp. nov., a spiral-shaped bacterium colonizing the mucus layer of the gastrointestinal tract of laboratory rodentsBronwyn R. Robertson,¹ Jani L. O'Rourke,² Brett A. Neilan,² Peter Vandamme,³ Stephen L. W. On,⁴ James G. Fox⁵ and Adrian Lee²^{1,2}Ramaciotti Centre for Gene Function Analysis¹ and School of Biotechnology and Biomolecular Sciences², The University of New South Wales, Sydney, NSW 2052, Australia³Laboratorium voor Microbiologie, Universiteit Gent, Ledeganckstraat 35, B-9000 Gent, Belgium⁴Danish Veterinary Laboratory, Bülowsvej 27, DK-1790 Copenhagen V, Denmark⁵Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

The mammalian gastrointestinal tract is covered by a layer of mucus that can harbour a range of bacterial species specifically adapted to colonize this ecological niche. Examination of 110 bacterial isolates cultivated from the gastrointestinal tract of 23 mice revealed the presence of a subgroup of 30 isolates that did not correspond genetically with genera commonly associated with this site, i.e. members of the *ε-Proteobacteria* such as *Helicobacter* and *Campylobacter* species. Instead this group of isolates was found to lie within the phylum *Deferribacteres*, a completely distinct lineage in the domain *Bacteria*. There was a high level of consensus in results obtained from the phenotypic and genotypic characterization of a number of the isolates, which showed they were distinct from other members of the *Deferribacteres*. As such, they are proposed to constitute a new genus and species, *Mucispirillum schaedleri* gen. nov., sp. nov. These organisms are anaerobic, Gram-negative, spiral-shaped rods with bipolar flagella. The type strain is HRI 117^T (= ATCC BAA-1009^T = ACM 5223^T).

INTRODUCTION

The layer of mucus that lines the mammalian gastrointestinal tract (GT) represents a complex ecological niche (Lee, 1980). We have previously hypothesized that bacteria capable of colonizing this site possess specialized characteristics, such as spiral morphology and motility, which give them a selective advantage over other bacterial types in viscous environments and that spiral bacteria have co-evolved with their mammalian hosts (Lee, 1985). For example, the mucus lining of the GT of rodents harbours

large and often diverse populations of spiral bacteria, many of which have been found to be members of the genera *Helicobacter* and *Campylobacter* (Davis *et al.*, 1972; Erlandsen & Chase, 1972; Gordon & Dubos, 1970; Lee *et al.*, 1968; Phillips *et al.*, 1978; Savage & Blumershteyn, 1974).

Recently, a major emphasis of research into GT disease has focused on the role such bacteria, specifically the spiral-shaped *Helicobacter* species, play in the disease processes. In addition to *Helicobacter pylori* being shown to be a causative agent in gastric disease, there is now accumulating evidence that many other *Helicobacter* species may be implicated in other gastrointestinal and hepatobiliary diseases, including enteritis, inflammatory bowel disease (IBD), typhlitis, hepatitis and hepatic neoplasia (Fox, 2002; O'Rourke *et al.*, 2001; Schauer, 2001). GT research relies on the mouse as an important animal model for studies on pathogenicity and underpins much of the current research on anti-*Helicobacter* vaccine development. However, our knowledge of the identity of the many spiral-shaped bacteria that are capable

Published online ahead of print on 4 January 2005 as DOI 10.1099/ijs.0.63472-0.

Abbreviations: GT, gastrointestinal tract.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of nine *Mucispirillum schaedleri* isolates are AF059186–AF059190 and AY387668–AY387671.

A dendrogram based on whole-cell protein profiles of ten *Mucispirillum schaedleri* isolates is available as supplementary material in IJSEM Online.

of colonizing the mammalian GT is limited and should not be restricted to *Helicobacter* species. Therefore, it has become a priority to determine the identity of spiral bacteria naturally occurring in laboratory rodents. Although many *Helicobacter* species have been identified in laboratory mice and rats (Fox *et al.*, 1994, 1995; Lee *et al.*, 1992; Mendes *et al.*, 1996; Robertson *et al.*, 2001; Schauer *et al.*, 1993; Shen *et al.*, 1997), it is becoming increasingly obvious that bacteria belonging to other, possibly unknown, genera are also capable of colonizing the mammalian GT. Such findings would have important implications on how the many models of GT disease are undertaken and interpreted.

During a survey examining spiral bacteria that naturally infect mice obtained from four animal facilities in Sydney, Australia, we cultured a large number of anaerobic spiral bacteria that did not belong to either the genus *Helicobacter* or the genus *Campylobacter*. This report presents the taxonomic characterization of these non-helicobacter isolates, including 16S rRNA gene sequence, whole-cell protein, biochemical and morphological analyses. Genotypic studies indicated that these isolates fell within the phylum *Deferribacteres*, which has no close phylogenetic relationship to the genus *Helicobacter* or to the class *Proteobacteria*. A specific PCR targeting a region of the 16S rRNA gene was developed to define and identify this novel bacterial group. Based on these results a new genus and species, *Mucispirillum schaedleri* gen. nov., sp. nov., is proposed in honour of Russell Schaedler, one of the early pioneers of the study of the bacteria of the intestinal tract of mammals and the first to isolate a member of this genus (ASF 457) as part of the altered Schaedler flora (Dubos *et al.*, 1965; Savage *et al.*, 1968; Schaedler *et al.*, 1965; Dewhirst *et al.*, 1999).

METHODS

Bacterial isolation and cultivation. Conventional or Specific Pathogen Free, Quackenbush/Swiss mice ($n=23$) were obtained from four separate animal facilities in Sydney, Australia. Routine screening for spiral bacteria was performed by culturing intestinal mucus scrapings and homogenized liver samples, as described by Robertson *et al.* (2001). Briefly, mouse intestinal mucus scrapings or homogenized livers were inoculated directly onto horse blood agar (HBA) and *Campylobacter* selective agar (CSA). Alternatively, the specimens were first inoculated onto 0.65 µm filters (Millipore) on the agar surface of plates and placed in a 37 °C incubator (10 % CO₂ and 95 % humidity) for 2 h. The filters were then removed and discarded. All plates were incubated, lids uppermost, in an anaerobic jar (HP 11; Oxoid) with a microaerobic gas-generating kit (BR 56; Oxoid) or with an anaerobic gas-generating kit (BR 38; Oxoid) at 37 °C for 72–96 h. After incubation, the plates were examined for growth of spiral bacteria using phase-contrast microscopy. Subcultures were carried out until pure cultures were obtained.

Electron microscopy. Fresh bacterial cultures were fixed in 1 % glutaraldehyde for 1 min, negatively stained with phosphotungstic acid (1 %) and viewed with a Hitachi H7000 transmission electron microscope.

DNA extraction, 16S rRNA gene amplification and sequencing. DNA was extracted from a loopful of cells using a modification of the bacterial lysis method of Tillett and Neilan (Robertson

et al., 2001; Tillett & Neilan, 2000). PCR amplification and sequencing of the 16S rRNA gene were performed as described by Robertson *et al.* (2001). In all cases, both strands of DNA were sequenced with contiguous overlaps.

Phylogenetic inferences. Sequences were aligned using the multiple sequence alignment tool in the CLUSTAL X package (Thompson *et al.*, 1997). Aligned sequences were corrected manually and nucleotide positions that contained ambiguities were removed from further analysis. Genetic distances, corrected for multiple base changes by the method of Jukes & Cantor (1969), were calculated using the DNADIST program in the PHYLIP package, version 3.57c (Felsenstein, 1989). The phylogenetic tree was reconstructed using the neighbour-joining method of Saitou & Nei (1987) and the tree plotted using NJPLOT in the CLUSTAL X package. Bootstrap analysis of 100 resamplings of the dataset, created using SEQBOOT, were determined using DNADIST and CONSENSE, also from the PHYLIP package (Felsenstein, 1985).

Phenotypic characterization. Five strains were examined using 65 of the tests included in an extensive identification scheme for *Campylobacter*, *Helicobacter* and related bacteria as described by On *et al.* (1996), except that strains were cultured under anaerobic conditions on BHI agar supplemented with 5 % blood prior to testing. Additional enzyme activities were examined in strain ASF 457 by use of a commercial identification kit (API 32E; bioMérieux). These tests were performed using the manufacturer's guidelines, except that the assays were performed under anaerobic conditions.

SDS-PAGE of whole-cell proteins. Whole-cell protein extracts were prepared and SDS-PAGE performed as described by Pot *et al.* (1994). The densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis were performed using the GELCOMP software package version 4.2 (Applied Maths), and the profiles were recorded and stored on a PC. The similarity between all pairs of traces was expressed by the Pearson product moment correlation coefficient and is presented as percentages of similarity.

RESULTS AND DISCUSSION

Isolation, growth characteristics and ultrastructure

In this study we present the isolation and classification of a group of anaerobic, spiral bacteria cultured from the intestines and livers of laboratory rodents and propose the construction of a new genus and species to accommodate them, *Mucispirillum schaedleri*. They constituted 27.3 % (30/110) of the spiral-shaped bacteria isolated in a study of such bacteria in mice. These isolates were found in 13/17 mice originating from three of the animal houses examined in this study, including the Microbiology animal facility at the University of New South Wales (UNSW). The bacteria were most commonly cultivated from caecal samples (14/30) but they could also be found in ileal, colonic and liver tissue samples (5/30, 10/30 and 1/30, respectively). Three additional isolates from our collection of spiral bacteria also fitted this profile. These isolates had been cultured previously from Quackenbush Swiss mice in 1995 and a Wistar rat in 1978 from animals also housed at the Microbiology animal facility, UNSW. Although most of the isolates were cultivated from the mucus layer covering the intestinal tract of the rodents, two of the isolates

originated from liver samples. This raises the possibility that in certain animals, especially older animals, these bacteria are capable of translocating from the intestinal tract to the hepatobiliary system. A similar occurrence has been seen with several *Helicobacter* species (Fox *et al.*, 1994, 1995; Stanley *et al.*, 1994).

When observed in wet mounts under phase-contrast microscopy these bacteria had a thin, tight spiral morphology. They were Gram-negative and not capable of growth under microaerobic conditions. Electron microscopic examination of a number of isolates revealed that they possessed single, bipolar, unsheathed flagella (Fig. 1).

16S rRNA gene sequence analysis

The 16S rRNA gene sequence was determined for nine isolates. All nine sequences had between 96 and 100 % sequence similarity with one another. Sequences obtained from four isolates (HRI I01, HRI I10, HRI I12 and HRI I17^T) had 100 % sequence similarity with each other, while a further three isolates (UNSW I28, UNSW I26 and ABHU I23) also shared 100 % sequence similarity with each other. There were four nucleotide mismatches between these two groups. The remaining two isolates, UNSW I29 and UNSW I30, were slightly less closely related (96.6–97.1 %) to the main group. Phylogenetically similar bacteria were also found to have been isolated from a rodent and a Colobus monkey in the USA (strains ASF 457 and Lincoln Park 3, respectively) (Dewhirst *et al.*, 1999; Sarma-Rupavtarm *et al.*, 2004). The 16S rRNA gene sequence of strain ASF 457 was found to have 100 % similarity with four strains isolated in this study, including the nominated type strain, HRI I17^T. A phylogenetic tree was reconstructed from a multiple sequence alignment (1317 nt in length) of 16 16S rRNA gene sequences (Fig. 2). These included representatives of the four different sequences determined in this study (HRI I17^T, UNSW I26, UNSW I29 and UNSW I30) and representatives from the *Deferribacteraceae* that were the most closely related taxa returned in searches of sequences available in the GenBank database.

Fig. 1. Transmission electron micrograph of *Mucispirillum schaedleri* sp. nov., a spiral-shaped bacterium with bipolar flagella measuring 4 µm × 0.4 µm in size. Negative stain. Bar, 0.66 µm.

The phylum *Deferribacteres* is a relatively recently described lineage within the bacterial domain (Huber & Stetter, 2001). Four genera, each containing small numbers of species, are described in the *Deferribacteraceae*, *Flexistipes* (Fiala *et al.*, 1990), *Geovibrio* (Caccavo *et al.*, 1996), *Deferribacter* (Greene *et al.*, 1997) and *Denitrovibrio* (Myhr & Torsvik, 2000). The level of sequence similarity between our sequences described above and other bacteria in this group (80–85 %) did not support a generic classification.

Specific PCR development

Based on the comparison of 16S rRNA gene sequences obtained here with those from other bacterial groups, oligonucleotides were designed for use in a PCR to identify other bacterial isolates as members of this previously unrecognized bacterial group. Candidate primers were identified at nucleotide positions 137–158 and 1115–1134 (*Escherichia coli* numbering) and were designated MsF158 (5'-TTTAGACTGGAACAACCTACC-3') and MsR1115 (5'-CTATTTCCAGTTGCTAACGG-3'), respectively. The 16S rRNA gene sequence from the Colobus monkey isolate, Lincoln Park 3, did not show similarity to either MsF158 (six mismatches) or MsR1115 (two mismatches), nor did the other sequences representing members of the phylum *Deferribacteres*. PCRs were prepared as described above and the specific amplification of DNA from the nine sequenced isolates was achieved using the following thermocycling profile. An initial denaturation at 94 °C for 3 min was followed by 30 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min. Amplification was completed by a single extension step at 72 °C for 10 min. A range of DNA from other bacterial types served as negative controls. Using this PCR, all 33 isolates cultured on CSA under anaerobic conditions gave a positive result (data not shown).

Physiological and biochemical characterization

Five strains (ABHU I23, HRI I12, HRI I17^T, UNSW I26 and ASF 457) were further characterized with a comprehensive range of phenotypic tests. They were shown to produce oxidase and catalase. Strain ASF 457 grew only on BHI medium and not on the standard 5 % blood agar used as the base medium for growth tests. Similarly, only ASF 457 proved unable to grow on 5 % blood agar medium that contained cephalothin (32 mg l⁻¹), carbenicillin (32 mg l⁻¹) or cefoperazone (64 mg l⁻¹); the remaining four strains proved resistant to these antibiotics under the conditions examined. However, no positive results were observed among any of the five strains in the remainder of the conventional tests used to characterize them (see species description below for details). By use of the API 32E kit, acidification of the substrates L-arabitol, galacturonate and 5-ketoglutarate was consistently detected in strain ASF 457.

SDS-PAGE of whole-cell proteins

Whole-cell protein extracts from ten isolates (UNSW I24, UNSW I25, UNSW I26, UNSW I29, UNSW I30, HRI I01,

Fig. 2. Phylogenetic tree showing the position of *Mucispirillum schaedleri* sp. nov. based on analysis of near-complete 16S rRNA gene sequences derived from members of the *Deferribacteres* with *Chrysiogenes arsenatis* as the outgroup. Bar, 0.02 substitutions per site. Bootstrap values are indicated at the nodes.

HRI I10, HRI I12, HRI I17^T and ABHU I23) were subjected to SDS-PAGE. All ten isolates had very similar cellular protein profiles with similarity levels above 92.6% (see Supplementary Fig. A in IJSEM Online). The high degree of whole-cell protein similarity strongly suggests that all ten isolates examined represent a single species (Pot *et al.*, 1994). This is further endorsed by the extremely high level of 16S rRNA gene sequence similarity (>99.7%) among six of these isolates. The two isolates with slightly diverging 16S rRNA gene sequences (UNSW I29 and UNSW I30, ~96–97% similarity to the other isolates examined) cluster among the other isolates (Supplementary Fig. A), demonstrating that the observed differences in 16S rRNA gene sequences reflect intraspecies diversity. Although no DNA–DNA hybridization experiments were performed, the unique phylogenetic position (Fig. 2) and the high degree of consensus among the phenotypic and genotypic results indicate that it is appropriate to classify all ten murine isolates into a single novel species in a novel genus (Ursing *et al.*, 1995).

Prior to this study the majority of spiral-shaped bacteria identified in the mammalian GT were found to belong to the phylum *Proteobacteria* [genera *Helicobacter* and *Campylobacter*, of the ϵ -*Proteobacteria* (Murray *et al.*, 1990; Vandamme *et al.*, 1991), and the genus *Anaerobiospirillum* of the γ -*Proteobacteria* (On, 2001)]. In contrast, the phylogenetic position of *Mucispirillum*, the mucosa-associated spiral-shaped bacterium described in this report, is within the phylum *Deferribacteres*, a completely distinct lineage within the domain *Bacteria*. Members of the family *Deferribacteraceae* have been isolated from a range of diverse water-based environmental samples. These bacteria, including *Mucispirillum*, are all anaerobic but share few other phenotypic characteristics except for being curved rods, vibroid or, in the case of *Mucispirillum* isolates, spiral-shaped. The majority are motile and they also include thermophilic as well as mesophilic organisms.

A recent study by Paster *et al.* (2001) also raises the possibility that a genetically similar group of organisms may

be able to colonize the oral cavity of humans, a niche known to be colonized by other spiral-shaped bacteria. In this study bacterial diversity in subgingival plaque was examined by analysis of a 16S rRNA gene sequence clonal library derived from healthy individuals and those showing signs of periodontal disease. Eighty-six of the 2522 clones analysed fell within the phylum *Deferribacteres* and formed eight separate phylotypes (clusters of clone sequences that differed from known species by approximately 2% and were at least 99% similar to members of their cluster).

In addition to the 30 *Mucispirillum* isolates cultured for this study, the fact that single isolates had been cultured previously from rodents on several occasions both in Australia and in the United States over many years indicates that this group of organisms is not geographically restricted. The isolation of a phylogenetically closely related bacterium from a Colobus monkey is interesting and suggests that there may exist additional species within this genus colonizing the intestinal tract of a variety of mammals in a similar way as has been shown for the genus *Helicobacter*. The development of a specific PCR, as described here, will allow for preliminary screening studies to determine the distribution of these organisms in the GT and possibly the oral cavity of humans and a range of mammalian species. The significance of the genetic relatedness of *Mucispirillum* to other members of the family *Deferribacteraceae*, a range of unusual environmental isolates, is at present unclear. However, the selective pressure of viscous environments such as mucus and oil reservoirs has resulted in a common phenotypic trait, curved to spiral-shaped motile organisms.

Description of *Mucispirillum* gen. nov.

Mucispirillum (Mu.ci.spi'ril.lum. L. n. *mucus* mucus; N.L. dim. neut. n. *spirillum* a small spiral; N.L. neut. n. *mucispirillum* a small spiral rod of the mucus).

Gram-negative curved to spiral rods with single, un-sheathed, bipolar flagella. No spores formed. Anaerobic; no growth obtained under microaerobic or aerobic conditions. Phylogenetically the genus belongs to the phylum *Deferribacteres*.

The type species is *Mucispirillum schaedleri*.

Description of *Mucispirillum schaedleri* sp. nov.

Mucispirillum schaedleri (schaed'ler.i. N.L. gen. n. *schaedleri* of Schaedler, in honour of Russell Schaedler, one of the pioneers in the study of the bacteria of the intestinal tract of mammals).

Gram-negative curved to spiral rods (3–4 µm × 0.4 µm) with single, un-sheathed, bipolar flagella. No spores formed. Anaerobic growth at 37 °C; no growth obtained under microaerobic or aerobic conditions or at 42 °C. Does not readily form single colonies, and requires 3–5 days for growth on 5% HBA. Non-haemolytic. Bacterial growth is not pigmented and pitting of the agar medium is not seen.

Oxidase- and catalase-positive. Does not reduce nitrate, selenite or triphenyl-tetrazolium chloride. Hydrogen sulfide production in triple-sugar iron agar is not seen. Most (80%) strains grow on 5% blood agar medium containing 32 mg cephalothin l⁻¹, 32 mg carbenicillin l⁻¹ or 64 mg cefoperazone l⁻¹. Strains do not grow on tyrosine, casein, lecithin or minimal agar medium, or on media containing 32 mg nalidixic acid l⁻¹, 100 IU 5-fluorouracil, 0.05% sodium fluoride, 0.032% methyl orange, 0.005% basic fuchsin, 1.0% glycine, 2–4% NaCl, 0.1% potassium permanganate, 0.0001% sodium arsenite, 0.02–0.05% safranin, 0.0005% crystal violet, 0.01% janus green or 0.02% pyronin. Isolated from the gastrointestinal mucus of rodents.

The type strain, HRI 117^T (=ATCC BAA-1009^T=ACM 5223^T), was isolated from mouse caecal mucus.

ACKNOWLEDGEMENTS

Automated sequencing was performed at the UNSW Automated DNA Analysis Facility.

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