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Multilocus analysis reveals diversity in the genus *Tissierella*: Description of *Tissierella carlieri* sp. nov. in the new class *Tissierellia* classis nov.☆

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ABSTRACT

The genus *Tissierella* and its relatives *Tepidimicrobium*, *Soehngenia* and *Sporanaerobacter* comprise anaerobic Gram-positive bacilli classified along with Gram-positive cocci in a family with controversial placement designated as *incertae sedis* XI, in the phylum *Firmicutes*. We performed a top-down reappraisal of the taxonomy from the phylum to the species level within the genus *Tissierella*. Reconstruction of high-rank 16S rRNA gene-based phylogenies and their interpretation in a taxonomic purpose allowed defining *Tissierellia classis* nov. within the phylum *Firmicutes* while the frames of *Tissierellales* ord. nov. and *Tissierellaceae* fam. nov. have to be further strengthened. For species delineation in the genus *Tissierella*, we studied a population of clinical strains. Beside *Tissierella praeacuta*, a sub-population of five strains formed a clade in multilocus phylogenies (16S rRNA, *cpn60*, *tpi*, *recA* and *spo0A* genes). Data such as 16S rRNA gene similarity level, population structure, chromosome organization and murein type indicated that this clade corresponded to a novel species for which the name *Tissierella carlieri* sp. nov. is proposed, with type strain LBN 295^T = AIP 268.01^T = DSM 23816^T = CCUG 60010^T. Such an approach, associating a phylogenetic reappraisal of high-level taxonomic ranks with weak taxonomic structure and a population study for genus and species delineation is needed to strengthen the taxonomic frame of *incertae sedis* groups in the phylum *Firmicutes*.

Introduction

The history of the species *Tissierella praeacuta* is emblematic of the "fluidity" of bacterial taxonomy and nomenclature. This species, formerly named *Bacteroides praeacutus* by Tissier in 1908 was reclassified as *T. praeacuta* in the new genus *Tissierella* [7] together with *Clostridium hastiforme* owing to molecular evidences and despite apparent morphological differences [2]. The genus *Tissierella* currently includes three species, *T. praeacuta*, *Tissierella creatinini* and *Tissierella creatinophila*, all recovered from environmental samples [18,24,29,33], while *T. praeacuta* remains to date the only species reported from various clinical sources [5,12].

The genus *Tissierella* belongs to the phylum *Firmicutes* and to the order *Clostridiales* but its placement at lower ranks depends on the classification used, i.e., either to the family *Peptostreptococcaceae* ([21], http://www.bacterio.cict.fr) or to the family *incertae sedis* XI ([36], http://www.ncbi.nlm.nih.gov/Taxonomy/). Considering the phylogenetic classification proposed by the Greengenes taxonomy (formerly Hugenholtz taxonomy), *Tissierella* belongs to an operational taxonomic unit (OTU) named '*Peptostreptococcaceae*' that probably corresponded to a rank higher than that of the family. Significant reclassifications within the phylum *Firmicutes* have been published recently and concerned particularly bacteria with non-typical Gram-positive cell wall such as *Tenericutes* [36] and *Negativicutes* [37].

Abbreviations: BBA, Brucella blood agar; CC, clonal complexes; ML, maximum likelihood; MLSA, multilocus sequence analysis; OTU, operational taxonomic unit; ST, sequence types; TGY, trypticase/glucose/yeast extract.

☆ The nucleotide sequences of the internal fragment genes used in this analysis have been deposited in the GenBank database under accession numbers given in Table 1.

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Considering this general context, *Tissierella* spp., including Gram-variable bacteria, worth to be considered in a global taxonomic reappraisal from the phylum to the species level. The availability of a collection of 18 rod-shaped anaerobic clinical isolates phenotypically identified as *T. praeacuta* gave us the opportunity to propose here a top-down reevaluation of the corresponding taxonomic lineage. High taxa were replaced in the phylum *Firmicutes* by 16S rRNA gene-based phylogeny, whereas genus and species were delineated by multi-gene phylogeny genetic data, population structure, low-resolution genome organization, cell-wall structure, morphology, and metabolic traits.

Materials and methods

Bacterial strains, growth conditions, and phenotypic characterization

Clinical ($n = 18$) and reference ($n = 8$) strains included in this study are described in Table 1. Strains were grown at optimal conditions as specified for each species [18,24,26,40,42,45]. Morphological and biochemical characteristics were determined as described [27,30] and using API 20A (bioMérieux). Colony morphology and presumptive identification tests were observed on Brucella blood agar (BBA) under anaerobic conditions at 37 °C. Susceptibility to special-potency discs was performed as recommended (Rosco). Metabolic end products were assayed by quantitative gas chromatography [1]. Enzyme profiles were generated with Rapid ID 32A (bioMérieux). For further biochemical characterization, the strains were grown in trypticase/glucose/yeast extract (TGY) broth.

The cell wall ultrastructure of strains *Tissierella* sp. LBN 295^T, *T. praeacuta* ATCC 25539^T and *C. hastiforme* ATCC 33268^T was examined by electron microscopy using a Philips CM12 transmission electron microscope [3]. Peptidoglycan of the three strains was extracted from early-exponential-phase cells by a method adapted from Courtin et al. [11] by increasing the speed of the centrifugation steps to 150,000 × *g* for 30 min. Reduced muropeptides were separated by reverse phase HPLC and then analyzed by MALDI-TOF mass spectrometry with a Voyager DE STR mass spectrometer (Applied Biosystems) with α-cyano-4-hydroxycinnamic acid matrix, as previously described [11].

Molecular methods

DNA was extracted by using the QIAamp DNA mini kit (Qiagen) for PCR amplification of 16S rRNA gene (1500 bp), as previously described [16]. Four housekeeping genes (*tpi*, *recA*, *spo0A* and *cnp60*) were also amplified as described in Table 2. PCR products were sequenced on an automated sequencer ABI PRISM 3100 (Applied Biosystems). DNA in agarose plugs digested by I-*Ceu*I (New England Biolabs) were separated using a CHEF-DRIII apparatus (Bio-Rad) in a 0.8% agarose gel in 0.5× Tris–Borate–EDTA buffer at 5.1 V cm^{-1} and at 10 °C, as previously described [1]. Two conditions of separation were used: (1) 50–100 s for 36 h; (2) 90–150 s for 24 h. Three independent measurements allowed the estimation of mean sizes of each band by comparison with *Saccharomyces cerevisiae* chromosomes.

Sequence analysis and phylogeny

16S rRNA gene sequences of 228 strains and clones of *Firmicutes* and of other different phyla ($n = 599$) [31] were chosen in GenBank, in Ribosomal Database Project II (http://rdp.cme.msu.edu) and in Greengenes (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) databases. Sequences were selected for length >1200 nt and for <1% ambiguous positions. For genes encoding proteins, the alignments were codon-cut after translation with TRANSLATE (http://www.expasy.org). The size of the codon-aligned sequences is indicated in Table 3. The sequences were concatenated manually. All sequences were aligned using CLUSTALW [49] or using NAST [13] programs. Evolutionary distance was analyzed by Neighbor-Joining (NJ) from a DNADIST F84 matrix [20]. Bootstrap values were calculated after 1000 reiterations. Maximum likelihood (ML) was computed by PHYML, model GTR plus gamma distribution and invariant sites [23].

The isolates were assigned to sequence types (ST) and then to clonal complexes (CC) using eBURST v3 [19]. Decomposition analysis of allelic profiles was represented by NeighborNet (SplitsTree 4.0) [28]. Genetic population analysis was performed using LIAN 3.1 [25] and SNAP software [34].

Results

High taxonomic rank phylogeny

The phylogenetic tree representing 159 different taxonomic units in the phylum *Firmicutes* is shown in Figs. 1 and S1. The same dataset was analyzed associated with datasets representative of the main other described bacterial phyla [31] (data not shown). Whatever the phylogenetic method used, members of the genus *Tissierella* appeared always included in the phylum *Firmicutes* whereas each described phylum included in the analysis formed a branch independent to each other. Within *Firmicutes*, the classes *Bacilli* and *Negativicutes* appeared as deep-branched clades (Figs. 1 and S1). As previously described [36,37], the class *Clostridia* was polyphyletic. *Tissierella* spp. formed a clade (bootstrap value: 80%) with the genera *Anaerococcus*, *Finegoldia*, *Helcococcus*, *Murdochiella*, *Parvimonas*, *Peptoniphilus* and *Soehngenia*, representing the first robust node after the phylum delineation (Fig. S1). These genera, that formed the family *incertae sedis* XI [36], were branched as deep as *Bacilli* and *Negativicutes*. The *incertae sedis* XI clade belonged to a larger OTU named '*Peptostreptococcaceae*' according to the Greengenes classification (Fig. 1) and supported by low bootstrap values. Members of *Peptostreptococcaceae sensu stricto* [36], i.e., *Tepidibacter*, *Peptostreptococcus*, *Filifactor* and *Sporacetigenium*, formed a far remote branch outside of the *incertae sedis* XI (Fig. S1). The ML trees in Figs. 2 and S2 showed phylogenetic relationships inside the *incertae sedis* XI. Members of the genus *Tissierella* and of the related genera *Soehngenia*, *Sporanaerobacter* and *Tepidimicrobium* formed a monophyletic clade in the distance trees (Figs. 2 and S2) but this clade was not robustly branched in the ML tree based on type strains (Fig. 2). Particularly, the monophyly of this group in ML phylogeny depended on the dataset used. These trees showed the delineation of other moderate to highly robust phylogenetic groups containing mainly Gram-positive cocci. All strains and clones named *Peptostreptococcus* sp., wrongly affiliated to the family *Peptostreptococcaceae sensu stricto*, branched outside of the *incertae sedis* XI clade (Fig. S1). The sequences of *Sedimentibacter* spp. and related clones, as well as the sequence of *Dethiosulfatibacter aminovorans*, uncertainly affiliated to the family *incertae sedis* XI ([36]; Ribosomal Database Project II), formed independent branches (Figs. 2, S1 and S2).

Genetic diversity within the genus Tissierella

The tree in Fig. 2 presented the phylogenetic relationships among the type strains of the genus *Tissierella* and related genera within the clade *incertae sedis* XI. The genus *Tissierella* appeared monophyletic but this structure depended on the dataset used. For instance, the ML tree presented in Fig. S2 that included clones and strains representative of the whole clade *incertae sedis* XI showed a paraphyletic structure of the genus. This was confirmed by a 16S