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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*.

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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-CeuI (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*, *Murdochella*, *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

Table 1
Characteristics of strains used in this study and results from genomic and genetic analysis.

Species	Strain No. ^a	Origin ^b (isolation site)	Phylo-genetic clade	Genomic size (Mb) (No. of <i>rrn</i> copies)	Sequence type	Clonal Complex ^c	Accession No.					
							16S rRNA gene	<i>cpn60</i>	<i>tpi</i>	<i>recA</i>	<i>spo0A</i>	
<i>T. praeacuta</i> / <i>C. hastiforme</i> (phenotypic identification)	AIP 224.02	H (skin and soft tissue)	A	3.25 (4)	1	S	GQ461802	HQ258937	GQ470975	GQ470946	GQ470923	
	AIP 30.02	H (blood)	A	ND	4	S	GQ461803	HQ258939	GQ470976	GQ470947	GQ470925	
	AIP 310.01	H (skin and soft tissue)	A	3.24 (4)	5	1	GQ461804	HQ258940	GQ470977	GQ470948	GQ470926	
	AIP 443.98	H (unknown)	A	3.29 (4)	6	1	GQ461805	HQ258941	GQ470978	GQ470949	GQ470927	
	AIP 488.98	H (skin and soft tissue)	A	ND	7	1	GQ461806	HQ258942	GQ470979	GQ470950	GQ470928	
	LBN 290	H (skin and soft tissue)	A	3.30 (4)	9	1	GQ461807	HQ258938	GQ470980	GQ470951	GQ470924	
	LBR 1	H (blood)	A	3.38 (4)	12	1	GQ461808	HQ258944	GQ470981	GQ470952	GQ470929	
	LBN 296	H (blood)	A	3.41 (4)	14	S	GQ461809	HQ258943	GQ470982	GQ470953	GQ470930	
	VPI 10426	A (central nervous system)	A	3.56 (4)	16	1	GQ461813	HQ258945	GQ470983	GQ470954	GQ470931	
	VPI 11497A	H (blood)	A	3.30 (4)	17	1	GQ461810	HQ258946	GQ470984	GQ470955	GQ470932	
	VPI 12486A	H (skin and soft tissue)	A	ND	18	1	GQ461811	HQ258947	GQ470985	GQ470956	GQ470933	
	AIP 249.98	H (osteo-articular)	B	ND	2	S	GQ461815	HQ258951	GQ470986	GQ470957	HQ258961	
	AIP 64.01	H (blood)	B	5.36 (4)	8	2	GQ461817	HQ258949	GQ470988	GQ470959	GQ470935	
	LBN 292	H (dacryocystitis)	B	5.39 (4)	11	2	GQ461819	HQ258948	GQ470990	GQ470961	GQ470937	
	LBN 295 ^T	H (skin and soft tissue)	B	5.75 (4)	13	2	GQ461821	HQ258950	GQ470992	GQ470963	GQ470938	
	IP Prévot 3583	H (unknown)	B	5.40 (4)	15	2	GQ461822	HQ258952	GQ470993	GQ470964	GQ470939	
	AIP 285.00	H (blood)	–	4.36 (4)	3	S	GQ461816	HQ258953	GQ470987	GQ470958	GQ470934	
	LBN 291	H (skin and soft tissue)	–	5.58 (6)	10	S	GQ461818	HQ258954	GQ470989	GQ470960	GQ470936	
	<i>C. hastiforme</i>	ATCC 33268 ^T	H (abdominal abscess)	A	3.36 (4)	19	1	GQ461814	HQ258935	GQ470973	GQ470944	GQ470921
	<i>T. praeacuta</i>	ATCC 25539 ^T	H (skin and soft tissue)	A	3.27 (4)	20	S	GQ461812	HQ258936	GQ470974	GQ470945	GQ470922
<i>T. creatinini</i>	DSM 9508 ^T	E (anaerobic sludge)	–	2.41 (4)	21	S	GQ461824	HQ258955	GQ470972	GQ470965	GQ470941	
<i>T. creatinophila</i>	DSM 6911 ^T	E (anaerobic sludge)	–	3.80 (2)	ND	ND	GQ461823	HQ258956	HQ258963	ND	GQ470940	
<i>C. ultunense</i>	DSM 10521 ^T	E (anaerobic sludge)	–	3.68 (4)	22	S	GQ461825	HQ258957	GQ470971	GQ470966	HQ258962	
<i>S. acetigenes</i>	DSM 13106 ^T	E (anaerobic sludge)	–	3.56 (4)	23	S	GQ461827	HQ258958	GQ470968	GQ470967	GQ470943	
<i>S. saccharolytica</i>	DSM 12858 ^T	E (anaerobic sludge)	–	ND	ND	ND	GQ461828	HQ258960	GQ470969	HQ258964	ND	
<i>T. ferriphilum</i>	DSM 16624 ^T	E (freshwater hot spring)	–	2.45 (4)	24	S	GQ461826	HQ258959	GQ470970	HQ258965	GQ470942	

ND, not determined.

^a AIP, Anaérobies Institut Pasteur; ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; LBN, Laboratoire de Bactériologie de Nancy; LBR, Laboratoire de Bactériologie de Rouen; VPI, Virginia Polytechnic Institute.

^b H, human origin; A, animal origin; E, environmental origin.

^c Clonal complexes = sets of related strains sharing at least two identical alleles; S, singleton = ST differing from every other ST by more than two loci.

Table 2
Characteristics of housekeeping gene sequences analyzed.

Targeting protein (encoding gene)	Targeting primers			T_m (°C)	Fragment size (bp)	Ref.
	Name	Sequence (5'–3')	Concentration (μM)			
Chaperonin 60 (<i>cpn60</i>)	H279A	GAIHIIIGCIGGIGAYGGIACIAC	50	37	600	[22]
	H280A	YKIVKITICICRAAICCGGIGCYTT				
Triose phosphate isomerase (<i>tpi</i>)	<i>tpiA</i>	GCWGGWAAYTGGAAARATGMAYAA	100	60→50 ^a	501	[14]
	<i>tpiB</i>	TTWCCWGTWCCDATWGCCCADAT				
Recombinase A (<i>recA</i>)	<i>recA-f</i>	TTHATYGANGCHGARCAAYGC	100	55	360	[15]
	<i>recA-r</i>	CCWCCWGWGTHGTYTCNGG				
Stage 0 sporulation protein A (<i>spo0A</i>)	<i>spo0A-f</i>	GGIRTICIGCICAYATHAARGGITA	25	55	300	[4]
	<i>spo0A-r</i>	CATIGCDATRAAYTCISWRRTIGTIGGYTT				

^a With 1 °C decremental steps in cycles 1–10.

Table 3
Results of multilocus sequence analysis (MLSA).

Targeting gene	Aligned sequence size in bp (in amino acids)	G + C mol%		Genetic diversity (h)			dN/dS	No of polymorphic nucleotidic sites	No of polymorphic amino acid sites
		Clade A	Clade B	All strains ^a	Clade A	Clade B			
<i>cpn60</i>	527 (175)	33.63	33.25	0.9710	0.9103	0.9000	0.1022	227	69
<i>tpi</i>	489 (163)	30.48	33.54	0.9203	0.7949	0.4000	0.1544	145	31
<i>recA</i>	360 (120)	38.44	38.83	0.9746	0.9231	0.9000	0.0490	300	99
<i>spo0A</i>	273 (91)	34.40	37.36	0.7645	0.2949	0.0000	0.1165	102	26 ^b

^a Clinical and reference strains tested using the multilocus analysis.

^b In strain *T. praeacuta* ATCC 25539^T, a mutation (G94T) lead to the creation of a STOP codon and to a truncated protein Spo0A, explaining the absence of sporulation observed for this strain.

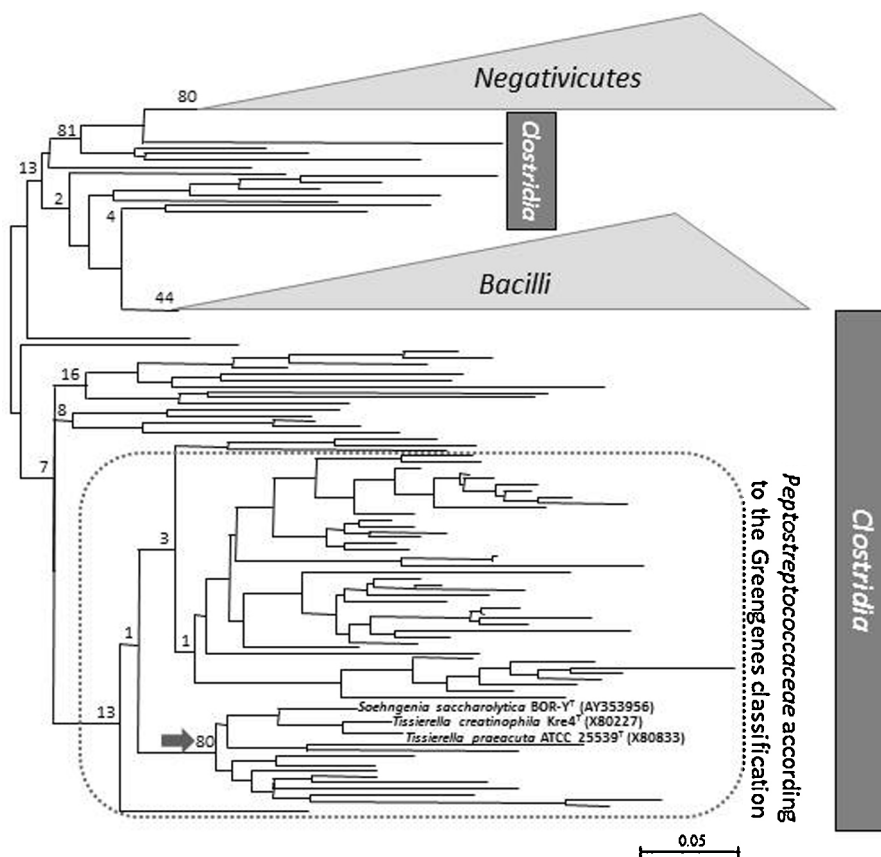


Fig. 1. Schematic representation of the ML tree based on partial 16S rRNA gene sequences (1127 nt) showing relationships between 159 members of the phylum Firmicutes. Numbers at nodes indicate percentages of bootstrap support, based on analysis of 100 replicates. They are indicated for the major nodes of interest. The root of the newly characterized *Tissierella* class (previously grouping *incertae sedis* XI family members) is indicated by an arrow. Members of the putative *Tissierellaceae* family are indicated in bold. The corresponding complete ML tree is available in Fig. S1. Bar, 0.05 substitutions per site.

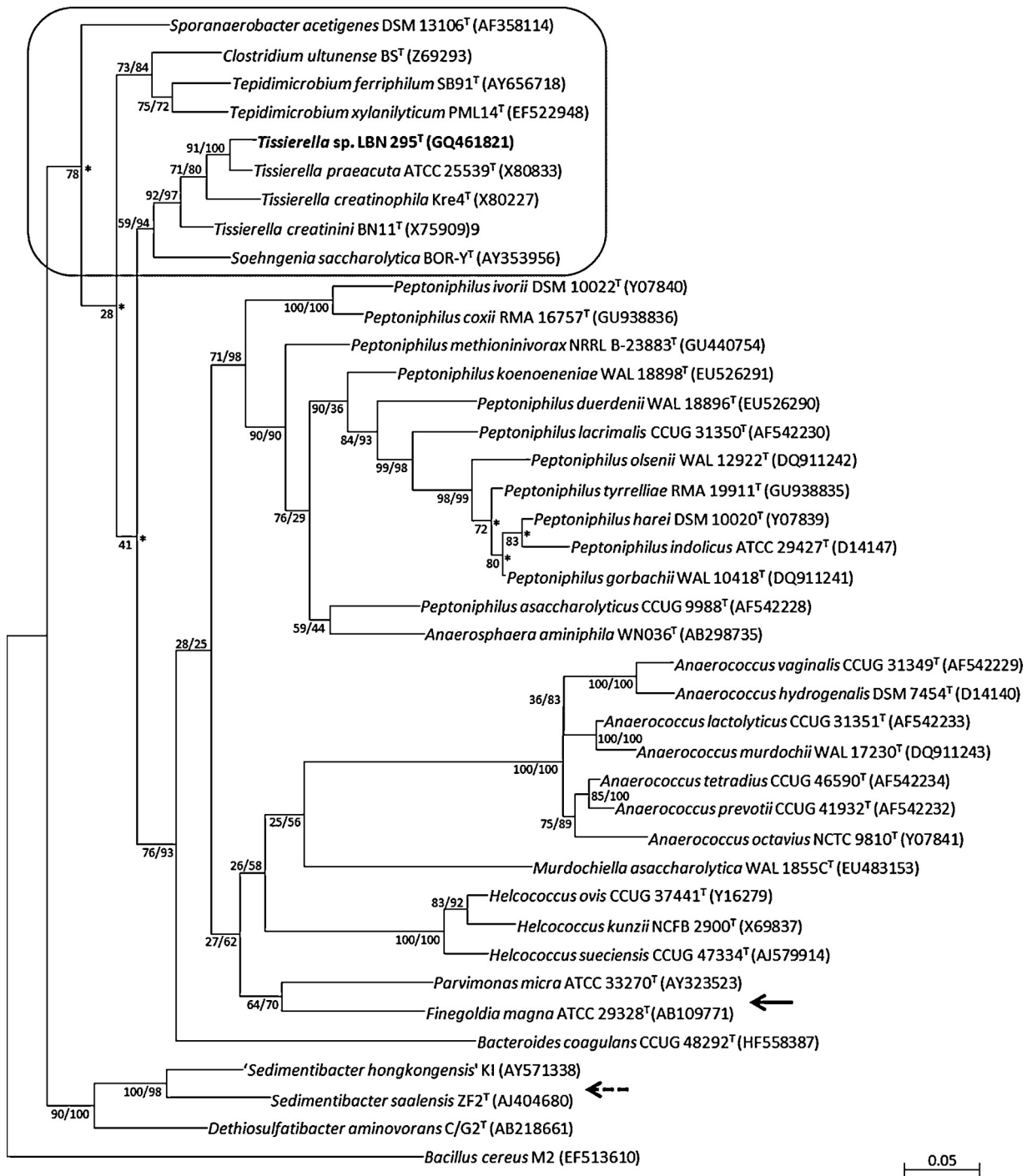


Fig. 2. ML tree based on partial 16S rRNA gene sequences (1268 nt), showing relationships between the 35 type strains of the newly characterized *Tissierella* classis nov., as well as type strains of the related genus *Sedimentibacter* and *Dethiosulfatibacter*. Type strain of the newly characterized *Tissierella carlieri* sp. nov. is indicated in bold. The species *Gallicola barnesae* DSM 3244^T and *Sedimentibacter hydroxybenzoicus* JW/Z-1^T were not included in the phylogenetic analyses due to poor quality of the 16S rRNA gene sequence available in the databases (NR_040843 and NR_029146, respectively): their phylogenetic placement in the tree when tested with the same dataset are respectively represented by a solid arrow and a dotted arrow. The *Bacillus cereus* 16S rRNA gene (EF513610) sequence was used as outgroup. Members of the putative family *Tissierellaceae* are inside the rectangle. This group, that appeared polyphyletic in this ML tree due to low bootstrap values, formed a monophyletic group supported by a bootstrap value of 97% in the corresponding NJ tree. Numbers at nodes indicate percentages of bootstrap support, based on analysis of 100 replicates (bootstrap value for ML tree/bootstrap value for NJ tree). Asterisks indicate nodes not conserved when another phylogenetic method (NJ) was used (the indicated bootstrap value correspond to ML method). Bar, 0.05 substitutions per site.

rRNA gene analysis on a dataset of sequences focused on clinical and type strains of *Tissierella* spp. and on related genera, i.e. *Soehngenia*, *Sporanaerobacter*, *Tepidimicrobium*, as well as on the species *Clostridium ultunense* (Fig. 3A). A similarity matrix constructed with 16S rRNA gene sequences showed two main groups of isolates phenotypically affiliated to *T. praeacuta*. The first one, named group A,

included the sequences of 11 isolates and those of *T. praeacuta* and *C. hastiforme* type strains sharing more than 99.9% of their nucleotide positions. Similarity group B contained five clinical isolates displaying more than 99.1% sequence identity. The sequences of group B strains differed by more than 5.9% of their nucleotide positions from most closely related *Tissierella* species. The higher level of

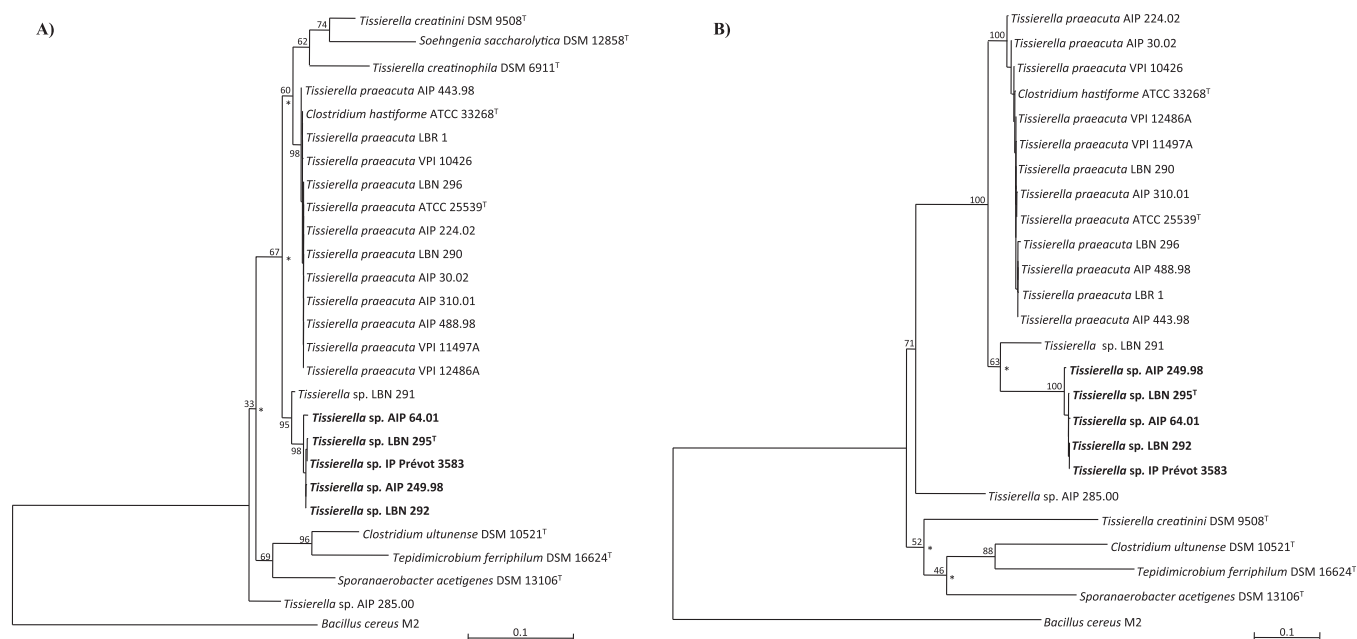


Fig. 3. ML trees based on (A) 26 partial 16S rRNA gene sequences (1415 nt), or (B) 24 concatenated partial sequences of genes *cpn60*, *tpi*, *recA* and *spo0A* (1646 nt), showing relationships between clinical and/or type strains of *Tisserella* spp. and of related genera. Members of the newly characterized *Tisserella carlieri* sp. nov. are indicated in bold. The *B. cereus* 16S rRNA gene sequence (EF513610) or concatenated *cpn60*, *tpi*, *recA* and *spo0A* from *B. cereus* BGSC 6E1 whole genome shotgun sequences were used as outgroups. Numbers at nodes indicate percentages of bootstrap support, based on analysis of 100 replicates. Asterisks indicate nodes not conserved when another phylogenetic method was used. Bar, 0.1 substitutions per site.

similarity between sequences of group A and group B strains was below 97%, thus a genetic diversity exceeding that of a single species can be assumed [48]. Particularly, the type strain of *T. praeacuta* ATCC 25539^T in clade A presented 96.6% sequence similarity with the strain *Tisserella* sp. LBN 295^T in clade B. The 16S rRNA gene-based phylogenies (Fig. 3A) showed clear delineation of two clades, A and B, corresponding to the similarity groups A and B, respectively.

The clinical isolates LBN 291 and AIP 285.00, which displayed respectively less than 98.6 and 93.8% 16S rRNA gene identity levels with members of clades A and B, formed independent lineages in the 16S rRNA gene-based trees (Figs. 3A and S2).

The sequences of the four genes *tpi*, *recA*, *spo0A* and *cpn60* were analyzed either independently or as concatenated sequences (1646 nt). We obtained a concatenated sequence for 24 strains (Table 1) among the 26 strains studied by 16S rRNA gene-based phylogeny in Fig. 3A. The phylogenetic tree based on concatenated sequences (Fig. 3B) was congruent with the 16S rRNA gene-based tree as well as with the four trees reconstructed with each independent gene (data not shown). The presence of two main clades supported by high bootstrap values was confirmed, type strains of *T. praeacuta* and *C. hastiforme* belonging to the clade A and strain LBN 295^T belonging to the clade B. Mean similarity values for the multi-locus concatenated sequences were 99.2% and 99.4% within clade A and clade B, respectively, and 88.3% between clades A and B. Again, strains LBN 291 and AIP 285.00 appeared as separated lineages.

The 24 strains used for multilocus sequence analysis (MLSA) showed 24 STs that were grouped using eBURST software into CCs that globally corresponded to clades A and B (Table 1). The mean genetic diversity at all loci (H) was high in the overall population ($H = 0.9076 \pm 0.0493$) whereas H decreased inside each clade ($H_{\text{cladeA}} = 0.7308 \pm 0.1481$; $H_{\text{cladeB}} = 0.5500 \pm 0.2179$). Genetic data about each gene of the multilocus analysis are given in Table 3. The diversity level decreased inside each clade for the genes *spo0A* and *tpi*. Consequently and as observed in the phylogenetic trees

reconstructed for each locus (data not shown), the *tpi* and *spo0A* genes gave the clearest delineation of clades and lineages inside the genus *Tisserella*.

Analysis of the linkage between alleles was carried out by determination of sI_A value. sI_A was significantly different from zero when all isolates were included in the analysis ($sI_A = 0.2061$; $p < 0.001$) but not when the analysis was performed for each group (clade A: $sI_A = 0.0431$, $p = 0.250$; clade B: $sI_A = 0.1605$, $p = 0.325$). Thus, the overall population displayed linkage disequilibrium, which was not observed inside each clade. Recombination events in the population are shown in Fig. 4. The graph generated clusters and branches consistent with previously recognized clades and phylogenetic independent lineages. It displayed a network-like structure, particularly observed for strains belonging to clade A and to a lesser extent between clade B isolates. Thus, recombination events occurred in both clades but clades A and B appeared genetically isolated without recombination between them, as also observed for other independent strains, including strains LBN 291 and AIP 285.00. This genetic isolation was congruent with the linkage disequilibrium observed in the overall population.

Genomic data

PFGE migration of intact DNA showed only one faint band suggesting that all the strains possessed a unique and circular chromosome (data not shown) [32]. The number of I-CeuI fragments and the estimation of chromosomal size deduced from I-CeuI fragment sizes addition are given in Table 1. These two genomic characters have been described as indicator of phylogenetic relationships between bacteria [35] and as data of taxonomic relevance [1]. Within each clade, all strains tested displayed similar restriction profiles while different genomic profiles were observed for clade A and clade B strains (Fig. S3). The major difference concerned a band of 2.3 Mb specific of clade B strains, the fragment with the highest molecular weight observed in profiles of clade A strains sizing no more than 1.2 Mb. The mean genomic size of clade

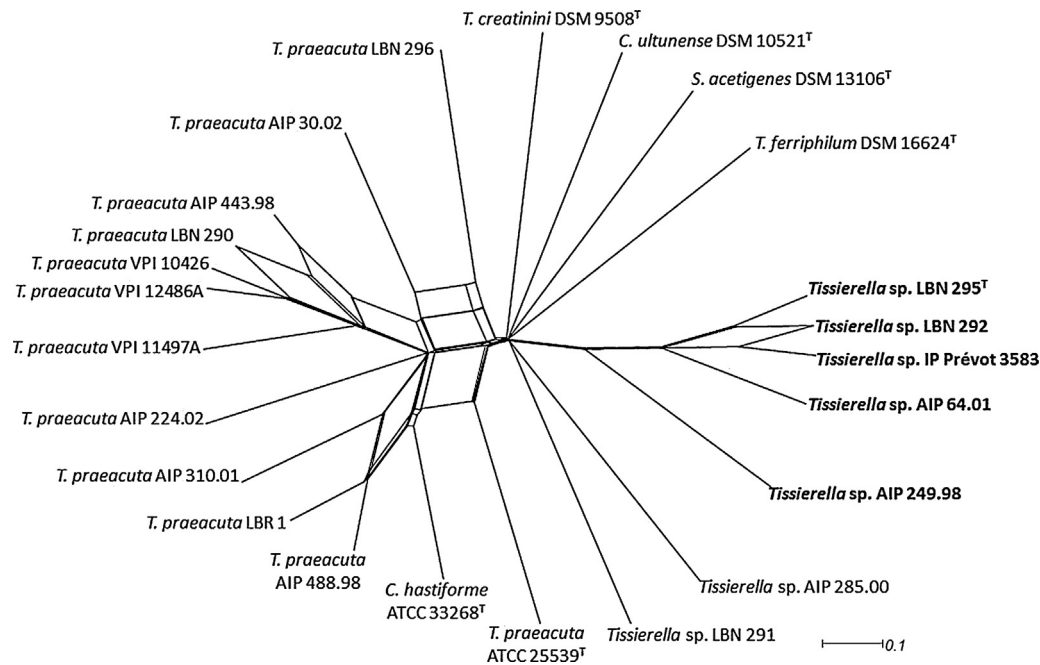


Fig. 4. SplitsTree decomposition analyses of multilocus data for 24 strains of *Tissierella* spp. and related genera. The distance matrix was obtained from allelic profiles of strains. Members of the newly characterized *Tissierella carlieri* sp. nov. are indicated in bold.

A strains was 3.34 Mb (± 0.10) whereas strains of clade B displayed a mean genomic size of 5.48 Mb (± 0.18). Pulsotypes observed for other strains differed from those observed for clade A and B strains (Table 1; Fig. S3).

Phenotypic characters of the genus *Tissierella*

Diversity of phenotypic characteristics observed among the population of clinical isolates is presented together with features for *T. praeacuta* ATCC 25539^T and *C. hastiforme* ATCC 33268^T in Table 4. Despite numerous phenotypic traits accounted for diversity in the population, only two enzymatic activities allowed differentiation between strains of clades A and B (PyrA and SerA). Characteristics that were common to all the strains were: rod-shaped, strictly anaerobic, no cell particular arrangement, following negative characteristics: catalase, urease activity, aesculin hydrolysis, indole, lecithinase and lipase production, asaccharolytic (acid not produced from arabinose, cellobiose, fructose, glucose, glycerol, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicine, sorbitol, sucrose, trehalose and xylose), and production of H₂S. Finally, susceptibility pattern to special-potency discs after 24 h of incubation was not discriminative among strains, all but one (strain LBN 291 susceptible to the colistin disk) showing susceptibility to vancomycin, kanamycin and metronidazole disks and resistance to colistin disk.

Transmission electron microscopic examination of ultrathin sections of two clade A strains (ATCC 25539^T and ATCC 33268^T) and one clade B strain (LBN 295^T) showed the presence of a Gram-positive cell wall structure with no outer membrane (Fig. S4). The peptidoglycan layer of these three strains was thinner (13–15 nm) than that of a typical Gram-positive cell (30–100 nm) [44]. After peptidoglycan extraction, the muropeptide profiles obtained by HPLC for *T. praeacuta* ATCC 25539^T and *C. hastiforme* ATCC 33268^T were identical to each other but differed from that obtained for strain LBN 295^T (Fig. S5). Composition analysis of peptidoglycan preparations for the three strains as well as major peaks observed on chromatograms for strains ATCC 33268^T and LBN 295^T confirmed the presence of the same amino acids, L-Ala, D-Glu and

mDAP, in agreement with the A1 γ structure previously described for *T. praeacuta* ATCC 25539^T [43] and *C. hastiforme* ATCC 33268^T [5]. The muropeptide profile similarity supports the taxonomic proximity of *T. praeacuta* ATCC 25539^T and *C. hastiforme* ATCC 33268^T whilst strain LBN 295^T shows a distinct profile that could be explained by differences in the activity of peptidoglycan synthesis, degradation and/or modification machineries.

Discussion

In this taxonomic study, we showed that a population of clinical isolates initially identified as *T. praeacuta* by phenotypic means was heterogeneous, its members being distributed in two distinct clusters. Five strains displaying homogeneous traits were affiliated to clade B and differed from the 11 members of clade A. The latter, containing the type strains of *T. praeacuta* and *C. hastiforme*, should be considered as the species *T. praeacuta sensu stricto*. Major arguments in favor of the affiliation of clade B strains to a new species in the genus *Tissierella* were: (1) 16S rRNA gene similarity level with other members of the genus under the recommended threshold of 97% (96.3–96.8% with the closest relative *T. praeacuta sensu stricto*) [48], (2) robust delineation using four-loci based phylogenies, (3) genetic exchanges in the sub-population that is isolated from other sub-populations, (4) difference in the muropeptide composition of peptidoglycan, (5) estimated chromosome size differing by more than 1.8 Mb (64% larger), in comparison with members of *T. praeacuta sensu stricto* and (6) phenotypic traits detailed in Table 4. The name *Tissierella carlieri* sp. nov. is proposed for this novel species with type strain LBN 295^T = AIP 268.01^T = DSM 23816^T = CCUG 60010^T.

Strain LBN 291 might represent a new species within the genus *Tissierella* whereas the belonging of strain AIP 285.00 to this genus was less clear. The phylotaxonomic relationships within the overall genus *Tissierella*, including *T. creatinini* and *T. creatinophila*, are unclear since, depending on the dataset, *Soehngenia*, *Tepidimicrobium*, *Sporanaerobacter* and *C. ultunense* could be branched inside the 16S rRNA gene-based phylogenetic frame of the genus *Tissierella*. All of these genera as well as the species *C. ultunense*

Table 4
Phenotypic characteristics variable among the population of 18 rod-shaped anaerobic clinical isolates, *T. praeacuta* ATCC 25539^T and *C. hastiforme* ATCC 33268^T.

Characteristic	Clade A strains			Clade B strains (n = 5)	Strain LBN 291	Strain AIP 285.00
	Clinical isolates (n = 11)	<i>T. praeacuta</i> ATCC 25539 ^T	<i>C. hastiforme</i> ATCC 33268 ^T			
Colony blackish pigmentation	+	–	+	+	+	–
Mobility	+	+	+	+	–	+
Gram staining	v*	–	+	v*	v*	v*
Spore formation	+	–	+	v	+	+
Gas production	v	–	–	v	–	+
Nitrate reduction	v	+	–	–	–	–
Gelatinase	+	+	+	+	+	–
Rapid ID 32A main results ^a						
PyrA	–	–	–	+	–	+
SerA	+	+	+	–	–	–
AlaA	v	+	+	v	–	–
Metabolic end products (range in mmol l ⁻¹) ^b						
Acetic acid	15.2–57.2	A	A	22.3–70.0	28.5	30.1
Propionic acid	3.2–14.4			3.2–6.9	3.4	5.8
Isobutyric acid	1.0–5.9			2.4–8.5	5.7	4.1
Butyric acid	3.9–22.6	B	B	14.5–36.8	26.5	45.5
Isovaleric acid	4.8–19.4	iV	iV	8.3–25.8	21.1	12.8
Lactic acid	(3.2–8.2)			(4.1–5.4)	–	5.3
2-Hydroxybutyric acid	(0.7)			(0.9–2.8)	0.6	–
Phenylacetic acid	2.1–9.6			2.4–10.0	4.0	8.2
Succinic acid	(2.0–3.7)			(2.8)	–	3.8

v, variable; v*, either Gram positive or Gram negative or variegated; R, resistant; S, susceptible.

^a PyrA, pyroglutamic acid arylamidase activity; SerA, serine arylamidase activity; AlaA, alanine arylamidase activity. All the clade A strains but two displaying poor profiles, showed weak positive reactions for the following activities: arginine arylamidase, leucine arylamidase, glycine arylamidase, histidine arylamidase, tyrosine arylamidase and phosphatase alkaline.

^b Main metabolic end products are indicated in bold type; A, B, iV indicated acetic acid, butyric acid and isovaleric acid as major end products for *T. praeacuta* ATCC 25539^T and *C. hastiforme* ATCC 33268^T [5,43]; inconstant production in a clade is indicated by parentheses; trace amounts (≤ 0.5 mmol l⁻¹) of other acids produced by a few strains were not indicated here; –, not detected.

grouped obligatory anaerobic Gram-positive or Gram-variable bacilli that represent a group independent from the Gram-positive cocci members (*Anaerococcus*, *Anaerosphaera*, *Finegoldia*, *Gallicola*, *Helcococcus*, *Murdochella*, *Parvimonas* and *Peptoniphilus*) of the current family *incertae sedis* XI (Figs. S1 and S2). However, their association in a new family as suggested by NJ phylogenetic reconstruction remains questioned due to low robustness depending on the dataset in ML phylogeny (Fig. 2). Characteristics that differentiate *T. carlieri* sp. nov. from other *Tissierella* species and from species of other related genera were presented in Table 5. The high taxonomic rank phylogenies reconstructed in this study confirmed that the current clade *incertae sedis* XI [36] belonged to the phylum *Firmicutes* and formed a class-level clade differing from the classes currently described in this phylum, i.e. *Clostridia*, *Bacilli* and *Negativicutes*. This suggested that the clade *incertae sedis* XI was a new independent class in the phylum *Firmicutes*, for which the name *Tissierellia* classis nov. is proposed.

Beside controversial delineation threshold ([47], for a review), a major and constant fact in the definition of species is that a species corresponds to a population of organisms or, for bacteria, a population of strains [47]. Paradoxically, and although the issue of the use of more than a single strain is raised at regular intervals [48], most of the bacterial species were described on the basis of only one individual strain and often one individual gene (i.e. 16S rRNA) considered as reflecting strain evolution. We developed for the first time a multilocus analysis based on four genes that could be used to study *Tissierella* spp. and closely related bacteria at the population level. The knowledge about these bacteria is scarce, particularly due to unavailable complete genome sequences. The multilocus analysis of the population gave arguments strengthening the description of a new species in the genus *Tissierella*. The genetic structure of the population highlighted the lack of major genetic exchanges between *T. praeacuta* and *T. carlieri*, arguing

for the genetic isolation of both sub-populations and thereby for their definition as two independent species. Although the bacterial species is merely a population concept, population structure was rarely used in bacterial taxonomy. We consider that it should be included in the polyphasic approach supporting the proposal for new bacterial species or for reappraisal of described taxa.

One of the best-described bacterial phyla in terms of diversity is *Firmicutes*, which is also one of the less robust in terms of taxonomy. New genera and species are frequently described in *Firmicutes* but the frame of the phylum remains weak and rather fluid. An effort is in progress using high-rank phylogenies to establish a stronger phylotaxonomy of *Firmicutes* [31,36,37]. High-rank taxonomy takes advantage of the robust phylogenetic definition of the phylum: the only phylogenetic character shared between members of two phyla is “belonging to the domain *Bacteria*”. Therefore, a group of bacteria forms a phylum if it cannot be placed in any taxon, except *Bacteria*. Efforts to replace a new low-rank taxon in a phylum can lead to the detection of clades those can be proposed as new classes or orders. With the description of *T. carlieri* sp. nov., we undertook a top-down approach from the phylum to the species. At this time, the class is the only level with robust phylogeny. The structuration in families and orders inside this class would be strengthened when wider species diversity will be available.

We proposed with this study a reappraisal of the *Firmicutes* classification by the creation of the new class *Tissierellia*. The corresponding class-level clade appeared as robust and low-branched as *Bacilli* and *Negativicutes*, two well-defined classes in the *Firmicutes*. The bacteria reclassified in *Tissierellia* classis nov. previously belonged to the class *Clostridia*, which is a paradigm of a non-cladistic catch-all taxon. A step-by-step exclusion of members of *Clostridia* with reclassification in robust new high-rank taxa seems to be an efficient way to clean up and strengthen the taxonomic framework of the phylum *Firmicutes*.

Table 5
Differential characteristics of *Tissierella* sp. LBN 295^T and related species. 1, *Tissierella* sp. LBN 295^T; 2, *Tissierella praeacuta*; 3, *Tissierella creatinini* DSM 9508^T [18]; 4, *Tissierella creatinophila* DSM 6911^T [24]; 5, *Clostridium ultimense* DSM 10521^T [42]; 6, *Soehingia saccharolytica* DSM 12858^T [40]; 7, *Sporanaerobacter acetigenes* DSM 13106^T [26]; 8, *Tepidimicrobium ferriphilum* DSM 16624^T [45]; 9, *Tepidimicrobium xylanilyticum* PML14^T [39].

Characteristic	1	2 ^a	3	4	5	6	7	8	9
Colonial morphology	Small, circular, entire, grayish-white	Small, circular, erose, grayish-white	nr	Small, circular, white to gray	Disk-shaped, whitish	Rhizoid, dark cream	nr	Spherical, brown	Round, whitish
Colonies blackish pigmentation	+	v	-	-	-	-	-	nr	nr
Mobility	+	+	-	-	+	+	+	nr	+
Gram staining	v*	v*	+	v*	-	v*	v*	v*	-
Spore formation	+	v	-	-	+	+	+	-	+
Production of:									
Nitrate reductase	-	v	-	-	-	-	-	-	+
Urease	-	-	-	+	nr	-	nr	nr	nr
Indole	-	-	-	nr	+	+	nr	nr	-
Gelatinase	+	v	-	nr	-	-	-	nr	-
H ₂ S	+	+	nr	nr	nr	+	+	+	+
Carbohydrates fermentation	-	-	-	nr	+	+	+	-	+
Major metabolic end product	A, B, iv	A, B, iv	A	A	A	A	A	A	A
Growth temperature range (°C)	20–45	20–39	20–39	10–40	15–50	15–40	25–50	26–62	25–67
Optimum growth temperature (°C)	37	37	37	30–34	37	30–37	40	50	60
Peptidoglycan structure type	Gram+	Gram+	nr	nr	Gram+	Gram+	Gram+	Gram+	Gram+
Genomic size (Mb)	5.36–5.75	3.24–3.56	2.41	3.80	3.68	nr	3.56	2.45	nr
Number of <i>rrn</i> copies	4	4	4	2	4	nr	4	4	nr

–, negative; + positive; v, variable; v*, either Gram positive or Gram negative or variegated; nr, not reported; A, acetic acid; B, butyric acid and IV, isovaleric acid.

^a From data obtained from all *T. praeacuta* strains included in this study and from the Bergey's Manual of Systematic Bacteriology [5,43].

Emended description of the genus *Tissierella* Collins and Shah 1986, emend. Farrow et al. 1995, emend. Bae et al. 2004

The characteristics of the genus are as described by Collins & Shah 1986, emend. Farrow et al. 1995, emend. Bae et al. 2004, except that terminal oval spores could be formed by some species. The genus *Tissierella* includes the validated species *T. creatinini*, *T. creatinophila*, *T. praeacuta*, and the proposed *T. carlieri* sp. nov. Genomic sizes range from 2.41 to 5.75 Mb with 4 *rrn* copies (except for *T. creatinophila* that possess 2 *rrn* copies).

The type species is *T. praeacuta* (Tissier, 1908) Collins and Shah 1986.

Description of *T. carlieri* sp. nov.

T. carlieri (car.li'e.ri. N.L. gen. n. *carlieri*, of Carlier, named in honor of Jean-Philippe Carlier, a French microbiologist at the Institut Pasteur, Paris, known for his research contributions to the taxonomy of the anaerobic bacteria).

Cells are motile, straight rods (1–4 μm × 0.5–0.8 μm in size) with rounded or occasionally tapered ends. After 48 h of incubation on BBA, colonies are small (0.7–1 mm in diameter), circular, entire, smooth, opaque, grayish-white. A blackish pigmentation could be observed after three to seven days of incubation. Growth occurs at 25–45 °C with an optimum at 37 °C. Cells are Gram-variable meaning that they may stain either negative or positive or variegated. No particular cells arrangement is observed. Terminal oval spores may or may not be produced but the sporulation-specific gene *spo0A* is present. Strictly anaerobic. Although susceptibility to vancomycin was observed after 24 h of incubation, heterogeneous resistance to vancomycin could be observed after prolonged incubation of the special-potency tablet assay. Catalase-negative. Asaccharolytic. Gas may be produced. Aesculin is not hydrolysed. Nitrates are not reduced. Indole and urease are not produced. Gelatin is digested. H₂S is produced. Lecithinase and lipase are not produced. Pyroglutamic acid arylamidase and inconstant alanine arylamidase activities are observed. Major metabolic end products in TGY broth are acetic, butyric, and isovaleric acids; smaller amounts of propionic, isobutyric, and phenylacetic acids are produced. Transmission electron microscopic examination of ultrathin sections of cells exhibits a Gram-positive cell wall structure without the presence of an outer membrane but with a very thin (13–15 nm) peptidoglycan layer. The cell wall murein is type A1γ (L-alanine and meso-diaminopimelic acid in first and third position respectively of the tetrapeptide and no interpeptidic bridge). Chromosomal genomic size ranges from 5.36 to 5.75 Mb, with four *rrn* operon copies.

The type strain is strain LBN 295^T (=AIP 268.01^T=DSM 23816^T=CCUG 60010^T) and reference strains are LBN 292, AIP 249.98, AIP 64.01 and IP Prévot 3583. Isolated from various human clinical sources (stump wound, dacryocystitis, bone abscess, blood).

Emended description of *T. praeacuta* (Tissier, 1908) Collins and Shah 1986

The description is modified as follows: Gram-stain-variable. The type strain shows alanine arylamidase and serine arylamidase activities in Rapid ID 32A. Chromosomal genomic size ranges from 3.24 to 3.56 Mb, with four *rrn* operon copies. Can be differentiated from other species of the genus *Tissierella* by sequencing 16S rRNA, *tpi* and *spo0A* genes.

Table 6
 Characteristics differentiating the genera of the class *Tissierellia*. 1, *Anaerococcus* [17,46]; 2, *Anaerospaera* [50]; 3, *Finogoldia* [38]; 4, *Gallicola* [17]; 5, *Helcococcus* [8–10]; 6, *Murdochella* [51]; 7, *Parvimonas* [38]; 8, *Peptoniphilus* [6,17,41,46,52]; 9, *Soehngenia* [40]; 10, *Sporanaerobacter* [26]; 11, *Tissierella* [7,18,24]; 12, *Tepidimicrobium* [39,45].

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Oxygen requirement	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic	Facultatively anaerobic	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic	Anaerobic aerotolerant	Anaerobic	Strictly anaerobic	Strictly anaerobic
Mobility	–	–	–	–	–	–	–	–	+ ^a	+	+	+
Cell morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Rods	Rods	Rods	Rods
Spores	–	+ ^b	–	–	–	ND	–	–	–	–	–	–
Utilization of:												
Peptones	+	+	+	+	ND	ND	+	+	–	+	–	+
Amino acids	+	+	+	ND	ND	ND	+	+	+	+	–	+
Carbohydrates	+	–	+	–	+	–	–	–	+	+	–	–
Major metabolic end products	B	A, B	A	A, L	L, A	L, OAA	A	A	A	A	A, B	A, B
DNA G + C content (mol%)	26–34	32.5	32–34	27–34	29.5–30	ND	27–29	25–35	43	32.2	28–32	33–36.2

v, variable; ND, not determined; wk, weak.

A, acetic acid; B, butyric acid; L, lactic acid; OAA, oxaloacetic acid.

^a Slightly motile in early phase of growth.

^b Thermotolerant cells.

Description of *Tissierellaceae* fam. nov.

Tissierellaceae (Tis.si.er.el.la'ce.ae. N.L. fem. n. *Tissierella*, type genus of the family; *-aceae*, ending to denote a family; N.L. fem. pl. n. *Tissierellaceae*, the *Tissierella* family).

The family *Tissierellaceae* includes the genera *Soehngenia* and *Tissierella*. The belonging of the validated genera *Sporanaerobacter*, *Tepidimicrobium* and of the currently misclassified *C. ultunense* to this family has to be further investigated.

As it was the first described, the type genus is the genus *Tissierella* (Tissier, 1908) Collins and Shah 1986, emend. Farrow et al. 1995, emend. Bae et al. 2004.

Description of *Tissierellales* ord. nov.

Tissierellales (Tis.si.er.el.la'les. N.L. fem. n. *Tissierella*, type genus of the order; *-ales*, ending to denote an order; N.L. fem. pl. n. *Tissierellales*, the *Tissierella* order).

The description is the same as for the family *Tissierellaceae* but the frame of this taxa has to be further strengthened.

The type genus is the genus *Tissierella*.

Description of *Tissierellia* classis nov.

Tissierellia (Tis.si.er.el'lia. N.L. fem. n. *Tissierella*, type genus of the type order of the class; *-ia*, ending to denote a class; N.L. neut. pl. n. *Tissierellia*, the *Tissierella* class). The class *Tissierellia* is defined in phylogenetic terms on the basis of 16S rRNA gene phylogeny. The class includes the validated genera *Anaerococcus*, *Anaerospaera*, *Finogoldia*, *Gallicola*, *Helcococcus*, *Murdochella*, *Parvimonas*, *Peptoniphilus*, *Soehngenia*, *Sporanaerobacter*, *Tepidimicrobium*, *Tissierella* and the misclassified species *Bacteroides coagulans* and *C. ultunense*. Members of the class (Table 6) harbor a Gram-positive cell wall structure and their reaction to Gram stain is positive or variable. The class includes obligatory anaerobic cocci and rods, and the facultative anaerobic genus *Helcococcus*. Aerotolerance may be noted. Motile or not. Endospores are formed or not. Mesophilic, rarely moderately thermophilic (genera *Sporanaerobacter* and *Tepidimicrobium*). Peptones and amino acids are the main sources of energy except for the genus *Tissierella*. Carbohydrates are fermented or not. Includes a few members with ability to reduce Fe(III) (*Tepidimicrobium*), to use inorganic sulfur-containing compounds like thiosulfate and/or elemental sulfur as electron acceptors (*Soehngenia*, *Sporanaerobacter* and *Tepidimicrobium*), and to survive to gamma-radiation exposure (*Tepidimicrobium ferriphilum*). Specific 16S rRNA signatures were not found.

Members of the class *Tissierellia* were found in various human and other animal microbiota, in methanogenic reactor and anaerobic digesters, and in environmental sources like freshwater hot spring. The class includes opportunistic human pathogens.

The type order is the order *Tissierellales* ord. nov.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.syapm.2013.09.007>.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.syapm.2013.09.007>.

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