Treponema peruense sp. nov., a commensal spirochaete isolated from human faeces

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Abstract

A Gram-stain-negative, obligatory anaerobic spirochaete (RCC2812^T) was isolated from a faecal sample obtained from an individual residing in a remote Amazonian community in Peru. The bacterium showed highest 16S rRNA gene sequence similarity to the pig intestinal spirochete *Treponema succinifaciens* (89.48%). Average nucleotide identity values between strain RCC2812^T and all available *Treponema* genomes from validated type strains were all <73%, thus clearly lower than the species delineation threshold. The DNA G+C content of RCC2812^T was 41.24 mol%. Phenotypic characterization using the API-ZYM and API 20A systems confirmed the divergent position of this bacterium within the genus *Treponema*. Strain RCC2812^T could be differentiated from the phylogenetically most closely related *T. succinifaciens* by the presence of alkaline phosphatase and α -glucosidase activities. Unlike *T. succinifaciens*, strain RCC2812^T grew equally well with or without serum. Strain RCC2812^T is the first commensal *Treponema* isolated from the human faecal microbiota of remote populations, and based on the collected data represents a novel *Treponema* species for which the name *Treponema peruense* sp. nov. is proposed. The type strain is RCC2812^T (=LMG 31794^T=CIP 111910^T).

Bacteria from the genus *Treponema* are typically anaerobic, spiral-shaped, highly motile micro-organisms that are fastidious to culture [1]. This genus comprises a number of primary pathogens responsible for syphilis [2] and periodontal disease [3] in humans, as well as digital dermatitis [4] in cattle. Commensal treponemas have received considerably less attention, although they are commonly found in the gastrointestinal tract of some insects [5] and mammals such as pigs and cows [6, 7], as well as most primates [8] including humans [9–12]. Next-generation sequencing data have shed more light on the taxonomic diversity and distribution of autochthonous *Treponema* members of the human gut microbiota, uncaptured by culturing methods. *Treponema* species have been consistently recognized as members of the faecal microbiome of humans living a traditional lifestyle, remote from industrialization, across continents, climates [11–13] as well as in extinct human populations (ancient humans from Mexico

[14]. To reveal any key functional role associated to these conserved gut microbes, characterization of cultured representatives is essential.

A previous cross-sectional metagenomic exploration revealed that *Treponema* was one of the dominant members of the gut microbiome of remote Matsés tribe populations living in small settlements along the rivers of the Peruvian Amazon. Subsequent genome reconstructions from that population suggested that these commensal *Treponema* strains fell outside the known pathogenic clades and were more similar to *Treponema succinifaciens* [15]. Here we report on strain RCC2812^T, recovered from a human stool sample as part of the Flora Intestinal Nativa project, an integrated study of the effect of industrialization on the gut microbiota of Peruvian populations (unpublished). On the basis of the information obtained from a polyphasic taxonomic approach, we propose

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Abbreviation: ANI, average nucleotide identity; DDH, DNA–DNA hybridization; FAA, fastidious anaerobe agar; FBS, foetal bovine serum; OrthoANI, orthologous average nucleotide identity; OTEB, oral *Treponema* enrichment broth.

The GenBank accession number for the 16S rRNA gene sequence of *Treponema peruense* RCC2812^T is MW159752. The GenBank accession number for the genome is CP064936.

Four supplementary tables and one supplementary figure are available with the online version of this article.

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Fig. 1. Phylogenetic analysis of 16S rRNA genes from all species of the genus *Treponema* presently recognized along with isolate RCC2812^T. This phylogenetic tree was generated with FastTree using the maximum-likelihood method, based on the generalized time-reversible model (there were a total of 1242 nucleotide positions in the final dataset). Bootstrap values (only those >70% are shown) based on 1000 replicates are shown at the branch nodes for the maximum-likelihood method. GenBank accession numbers are indicated in parentheses. Bar: 0,04 nucleotide substitutions per site.

that RCC2812^T represents a novel species within the genus *Treponema*.

ISOLATION AND ECOLOGY

Strain RCC2812^T was isolated from a human stool sample, collected from a seemingly healthy 29-year-old male resident of the Remoyacu village in the Peruvian Amazonian jungle, in the framework of the Flora Intestinal Nativa project (unpublished). The stool sample was collected within 30 min after defecation and stored in a 5 ml tube with 50% (v/v) of glycerol and frozen immediately at -80°C . It was transported to our lab on dry ice and further preserved at -80°C . 16S rRNA gene sequence analysis revealed that the sample was highly enriched in *Treponema* (30% of relative abundance). A sample aliquot of 200 mg (wet weight) was enriched in oral *Treponema* enrichment broth (OTEB; Anaerobe Systems) containing 10% foetal bovine serum (FBS; Gibco), 25 $\mu\text{g ml}^{-1}$ rifampin (Sigma-Aldrich) and 5 $\mu\text{g ml}^{-1}$ enrofloxacin (Sigma-Aldrich) for 24 h at 37°C in an anaerobic chamber ($\text{N}_2/\text{H}_2/\text{CO}_2$, 80:10:10, 37°C), as previously described [16–18]. The enriched culture was diluted in OTEB and plated on fastidious anaerobe agar (FAA; Lab M), supplemented with 5% defibrinated sheep blood (Sanbio), 25 $\mu\text{g ml}^{-1}$ rifampin

and 5 $\mu\text{g ml}^{-1}$ enrofloxacin. Single colonies were picked after 48–72 h, sub-cultured in the broth described above and selected for further characterization based on spiral shape cell morphology, determined using a phase contrast microscope. Isolates were preserved at -80°C in OTEB broth supplemented with 10% (v/v) FBS, 5% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol. Selected isolates were also subjected to alkaline lysis followed by thermal lysis (15 min at 95°C), 16S rRNA was then amplified by PCR using AccuPrime Taq DNA polymerase (Invitrogen) with primers fd1 (5'-AGAGTTT-GATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTG TTACGACTT-3') [19]. Primer rP2 was used for Sanger sequencing (Eurofins Genomics). The sequences obtained were taxonomically assigned using NCBI BLAST. One isolate tentatively allocated to the genus *Treponema*, RCC2812^T, was further characterized taxonomically.

16S rRNA GENE PHYLOGENY

High molecular weight DNA was isolated from RCC2812^T using the Puregene cell and tissue kit (Qiagen) according to the manufacturer's instructions. A standard SMRTbell library with 10 Kb insertions was generated, after which PacBio long reads were sequenced on the PacBio Sequel

Fig. 2. (a) Genome representation of strain RCC2812^T mapping from the outside to the inside of the circle: all predicted genes, cog, kegg, go, non-coding RNA, G+C content and G+C skew, (b) gene predictions with COG and (c) KEGG.

System (Pacific Biosciences) with SMRTbell Template Prep Kit 1.0-SPv3 (Pacific Biosciences). Library construction and sequencing was performed at Novogene. Sequencing reads were assembled into one circular chromosome representing the RCC2812^T genome with coverage >400×. In order to more accurately determine the evolutionary relationships of strain RCC2812^T with other *Treponema* species, we retrieved the 16S rRNA gene identified in its genome sequence. Four copies of the 16S rRNA gene were found in three distinct operons across the genome [1007688–1169243(+); 2239131–2240673(–) and 2467010–2468552(–)], they all share above 99% sequence identity. We performed a search of the 16S rRNA genes against the EzBioCloud 16S rRNA gene database [20], which identified *T. succinifaciens* DSM 2489T as the closest species with a similarity of 89.48% (for all four copies of the 16S rRNA gene of strain RCC2812^T). MUSCLE alignment of the 16S rRNA genes of all *Treponema* type strains along with one of the 16S rRNA copies of RCC2812^T (1167707–1169238_DIR+=accession MW159752) allowed a FastTree maximum-likelihood phylogenetic tree reconstruction (Fig. 1). This analysis suggested that isolate RCC2812^T probably represents a novel *Treponema* species most closely related to *T. succinifaciens* and falls within a clade of commensal *Treponema* originating from the gastrointestinal tract of different mammalian hosts. Similar results were obtained using the neighbour-joining method (see Fig. S1, available in the online version of this article).

We further confirmed that strain RCC2812^T was a novel species by computing the orthologous average nucleotide identity (OrthoANI [21]) between the genome of RCC2812^T

and the genome of the other *Treponema* species whenever available (see Table S1 for a list of all analyses performed on *Treponema* type strains). The ANI was consistently <73% against RCC2812^T across all pairwise comparisons, the highest being 72.2% with *T. succinifaciens* (see Table S2), well below the 95% threshold for species delineation. Similarly, genome-to-genome distance calculation [22] from the DSMZ culture collection (<http://ggdc.dsmz.de/>) corroborated that RCC2812^T was a new species, with all estimated DNA–DNA hybridization (DDH) values being below 23% (see details in Table S3).

GENOME FEATURES

The genome of RCC2812^T comprises one chromosome of 2738066 bp, with a G+C content of 41.24mol%, 2580 predicted genes and no detected plasmids. As a reminder, the closest type strain *T. succinifaciens* DSM 2489^T possesses one chromosome of 2897425 bp with a G+C content of 39.13mol%, 2786 predicted genes and a plasmid of 165572 bp [23]. Out of the 2580 predicted genes of RCC2812^T only 1190 have a match in the genome of *Treponema succinifaciens* DSM 2489^T according to the NCBI-BLAST non-redundant (nr) database, 2033 were functionally annotated using the NCBI-BLAST non-redundant database (version 201611), 1919 using KEGG (version 201801) and 1294 using COG (see details in Fig. 2).

To determine if genomes similar to strain RCC2812^T have been identified in other metagenomic datasets, we computed the average nucleotide identity of RCC2812^T against all the *Treponema* species genomes available in the Unified Human

Fig. 3. (a and b): Conventional scanning electron microscopy images of RCC2812^T (a, in spiral shape, b, in cystic form). (c): Transmission electron microscopy images of RCC2812^T with negative staining (arrow showing one of the two periplasmic fibrils originating subterminally from the end of the protoplasmic cylinder). (d): Conventional transmission electron microscopy image of a thin side section of a RCC2812^T cell (arrow showing again two periplasmic fibrils in bundle surrounded by the outer sheath).

Gastrointestinal Genome collection [24]. We identified 53 genomes with an average nucleotide identity above 95%, indicating this species is present in other metagenomic datasets (see Table S4). Interestingly, this species of *Treponema* was only identified in datasets from remote and rural unindustrialized populations: in Latin America (seven from a rural village in El Salvador [25], 29 from Peru [15, 25], Africa (five from rural communities in Madagascar [26], seven from Tanzania [13]) and Oceania (five from rural agrarian communities in the Fiji Islands [27], having the highest prevalence in Peru (around 11% of samples).

MORPHOLOGY AND PHYSIOLOGY

Cells of strain RCC2812^T stain Gram-negative and appear, under a phase contrast microscope, as highly motile with a helical coil. Using electron microscopy (see Fig. 3),

RCC2812^T exhibited all typical cell morphology features of the genus: small, helical spirochaete with four periplasmic flagella in a 2:4:2 arrangement. Cells were approximately 4–6 µm long and had a diameter of 0.2–0.3 µm with two to five spirals.

RCC2812^T displays good growth on FAA plates supplemented with 10% FBS and 5% defibrinated sheep blood at 37 °C under anaerobic conditions, but grows equally well without serum or blood. On the contrary, *T. succinifaciens* can grow on FAA plates without blood but grows poorly without serum. After 48 to 72 h on FAA, strain RCC2812^T produces punctiform translucent non-pigmented colonies with a diameter of approximately 0.5 mm, smooth surface and a slight cream colour. No local haemolysis was observed after 4 weeks of plate culture. The strain reaches stationary phase in unsupplemented OTEB within 72 h. The strain is mesophilic,

Table 1. Comparison of enzyme activity profiles of oral and gastrointestinal *Treponema* type strains from human, porcine and bovine hosts using the API ZYM system

Enzymes tested: 1, alkaline phosphatase; 2, C4 esterase; 3, C8 esterase lipase; 4, C14 lipase; 5, leucine arylamidase; 6, valine arylamidase; 7, cystine arylamidase; 8, trypsin; 9, chymotrypsin; 10, acid phosphatase; 11, naphtholphosphohydrolase; 12, α -galactosidase; 13, β -galactosidase; 14, β -glucuronidase; 15, α -glucosidase; 16, β -glucosidase; 17, *N*-acetyl- β -glucosaminidase; 18, α -mannosidase; 19, α -fucosidase. +, Positive; -, negative.

Species/subspecies	Strain	Presence of enzyme activity																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
<i>Treponema perniense</i>	RCC2812 ^T	+	-	-	-	-	-	-	-	-	+	+	-	+	-	+	-	-	-	-
<i>Treponema succinifaciens</i>	DSM 2489 ^T	-	-	+	-	-	-	-	-	-	+	+	-	+	-	-	+	-	-	-/+
<i>Treponema rectale</i> *	DSM 103679 ^T	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
<i>Treponema ruminis</i> †	DSM 103462 ^T	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-
<i>Treponema parvum</i> †	ATCC 700770 ^T	+	+	+	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-
<i>Treponema berlinense</i> ‡	ATCC BAA-909 ^T	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
<i>Treponema porcinum</i> ‡	ATCC BAA-908 ^T	-	+	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-
<i>Treponema pedis</i> §	DSM 18691 ^T	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
<i>Treponema medium</i> §	ATCC 700293 ^T	+	+	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
<i>Treponema brennaborensis</i>	DSM 12168 ^T	+	+	+	-	-	-	-	-	+	+	+	-	+	-	+	-	+	-	-
<i>Treponema pectinovorum</i> ¶	ATCC 33768 ^T	-	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-
<i>Treponema socranskii</i> subsp. <i>Socranskii</i> ¶	ATCC 35536 ^T	+	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-
<i>Treponema socranskii</i> subsp. <i>Buccale</i> ¶	ATCC 35534 ^T	+	+	+	-	-	-	-	-	+	+	+	-	-	+	-	-	-	-	-
<i>Treponema socranskii</i> subsp. <i>Paredis</i> ¶	ATCC 35535 ^T	+	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-
<i>Treponema maltophilum</i> ¶	ATCC 51939 ^T	+	+	+	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	+
<i>Treponema amylovorum</i> #	ATCC 700288 ^T	+	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	+
<i>Treponema denticola</i> **	ATCC 35405 ^T	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
<i>Treponema putidum</i> **	ATCC 700334 ^T	+	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+	-	-	-
<i>Treponema lecithinolyticum</i> ††	ATCC 700332 ^T	+	+	+	-	-	-	-	-	+	+	+	-	+	+	-	-	+	-	+

API ZYM data previously reported by *Evans et al. [18]; †Wyss et al. [28]; ‡Nordhoff et al. [29]; §Evans et al. [30]; ||Schränk et al. [31]; ¶Wyss et al. [32]; #Wyss et al. [33]; **Wyss et al. [34]; ††Wyss et al. [35].

with growth temperatures ranging from 20–37°C. Strain RCC2812^T is catalase-negative.

Further phenotypic characterization using the API ZYM system (bioMérieux) confirmed that strain RCC2812^T represents a phenotypically distinct species within the genus *Treponema* (see Table 1). The API ZYM test inoculum was prepared by harvesting cells from three fully grown FAA plates supplemented with 10% FBS and 5% sheep blood incubated for 48 h. API ZYM test series were determined four times following the manufacturer's instructions. Of all tested activities, only alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase and α -glucosidase activities were positive. As API ZYM data for the phylogenetically closest species *T. succinifaciens* DSM 2489^T were not previously reported, this strain was also included in the API ZYM test series. Strain DSM 2489^T exhibited esterase lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, β -glucosidase and slight α -fucosidase activities; all other reactions were negative.

In addition, strain RCC2812^T was also compared to *T. succinifaciens* DSM2489^T using API 20A and API rapid ID32 test series (bioMérieux) following the manufacturer's instructions. Bacterial inocula were prepared by harvesting cells from three FAA fully grown plates per strain supplemented with 10% FBS and 5% sheep blood after 48 h of incubation. In API 20A, strain RCC2812^T was positive for acidification of D-glucose, lactose, sucrose, maltose and D-mannose. On the contrary, *T. succinifaciens* DSM2489^T was also positive for acidification of maltose but not of D-glucose, lactose, sucrose and instead it was positive for D-xylose and cellobiose.

Using API rapid ID32, RCC2812^T was positive for β -galactosidase, β -galactosidase-6-phosphate and α -arabinosidase. *T. succinifaciens* DSM 2489^T shared only β -galactosidase activity with RCC2812^T. Contrary to RCC2812^T, *T. succinifaciens* DSM 2489^T had in addition α -glucosidase and β -glucosidase activities.

To determine the main fermentation products of strain RCC2812^T, a few colonies were harvested from FAA plates supplemented with 10% FBS and incubated in unsupplemented OTEB broth for 48 h. The culture was then centrifuged for 10 min at 2000 r.p.m. The resulting supernatant was used for HPLC analysis using Aminex HPLC columns (Bio-Rad Laboratories). *T. succinifaciens* DSM2489^T was used for reference, and OTEB medium as negative control. The major fermentation products of both *Treponema* strains were formate and acetate, followed by succinate and lactate that were produced in smaller amounts.

DESCRIPTION OF *TREPONEMA PERUENSE* SP. NOV.

Treponema peruense (pe.ru.en'se. N.L. neut. adj. *peruense* pertaining to Peru, where the sample from which the novel species was isolated came from).

Cells are Gram-stain-negative and highly motile under phase contrast microscopy. Under electron microscopy, cells appear as small, helical spirochaetes with four periplasmic flagella in a 2:4:2 arrangement, 4–6 μ m long and with a diameter of 0.2–0.3 μ m, and with two to five spirals. Strain RCC2812^T is a strict anaerobe that reaches stationary phase in unsupplemented OTEB medium after 72 h. When grown on FAA plates supplemented with 10% FBS at 37°C under anaerobic conditions for 48 h, it produces punctiform translucent non-pigmented colonies with a diameter of approximately 0.5 mm, smooth surface and a slight cream colour. In the API-ZYM system, strain RCC2812^T exhibited the following enzymatic activities: alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase and α -glucosidase. In API 20A assays, strain RCC2812^T tested positive for acidification of D-glucose, lactose, sucrose, maltose, D-mannose, raffinose and L-arabinose. Major fermentation products formed in OTEB medium were formate, acetate, succinate and lactate. The genome of this strain comprises one chromosome of 2738066 bp, with a G+C content of 41.24 mol%, 2580 predicted genes and no detected plasmid. On the basis of polyphasic taxonomic data presented here, we suggest recognition of RCC2812^T as a novel species within the genus *Treponema* for which the name *Treponema peruense* is proposed. The type strain is RCC2812^T (=LMG 31794^T=CIP 111910^T) and was isolated from a faecal sample obtained from an individual residing in a remote Amazonian community in Peru.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

The protocol for this study was reviewed and approved by the Ethics Committee of the Peruvian National Institute of Health (protocol OEE-010-15), who provided periodic monitoring during the execution of the project.

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