Stomatobaculum longum gen. nov., sp. nov., an obligately anaerobic bacterium from the human oral cavity

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A strictly anaerobic Gram-stain-variable but positive by structure, non-spore-forming bacterium designated *Lachnospiraceae* bacterium ACC2 strain DSM 24645^T was isolated from human subgingival dental plaque. Bacterial cells were 4–40 µm long non-motile rods, often swollen and forming curved filaments up to 200 µm. Cells contained intracellular, poorly crystalline, nanometre-sized iron- and sulfur-rich particles. The micro-organism was able to grow on yeast extract, trypticase peptone, milk, some sugars and organic acids. The major metabolic end-products of glucose fermentation were butyrate, lactate, isovalerate and acetate. The growth temperature and pH ranges were 30–42 °C and 4.9–7.5, respectively. Major fatty acids were C_{14:0}, C_{14:0} DMA (dimethyl aldehyde), C_{16:0}, C_{16:1ω7c} DMA. The whole-cell hydrolysate contained *meso*-diaminopimelic acid, indicating peptidoglycan type A1γ. The DNA G+C content was calculated to be 55.05 mol% from the whole-genome sequence and 55.3 mol% as determined by HPLC. There were no predicted genes responsible for biosynthesis of respiratory lipoquinones, mycolic acids and lipopolysaccharides. Genes associated with synthesis of teichoic and lipoteichoic acids, diaminopimelic acid, polar lipids and polyamines were present. According to the 16S rRNA gene sequence phylogeny, strain DSM 24645^T formed, together with several uncultured oral clones, a separate branch within the family *Lachnospiraceae*, with the highest sequence similarity to the type strain of *Moryella indoligenes* at 94.2%. Based on distinct phenotypic and genotypic characteristics, we suggest that strain DSM 24645^T represents a novel species in a new genus, for which the name *Stomatobaculum longum* gen. nov., sp. nov. is proposed. The type strain of *Stomatobaculum longum* is DSM 24645^T (=HM-480^T; deposited in BEI Resources, an NIH collection managed by the ATCC).

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Abbreviations: DAP, diaminopimelic acid; DMA, dimethyl aldehyde; FAME, fatty acid methyl ester; HMP, Human Microbiome Project; RAST, Rapid Annotation Subsystem Technology.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DSM 24645^T is HM120209. The complete high-quality draft genome sequencing was performed by the Broad Institute of Harvard and MIT as a part of *Lachnospiraceae* bacterium Group Sequencing Project, and available at <http://www.broadinstitute.org/>. The GenBank/EMBL/DDBJ assembled genome sequence of strain DSM 24645^T is AGEL01000000.

A supplementary figure and table are available with the online version of this paper.

The Human Microbiome Project (HMP) aims to characterize microbial communities inhabiting the human body and understand their role in health and disease (<http://www.hmpdacc.org/>; <http://commonfund.nih.gov/hmp/>). The oral cavity is a major gateway to the human body and one of the principal sites of interest to the HMP (Dewhirst *et al.*, 2010). Novel anaerobic species are of particular importance as they comprise approximately one half of the human oral microbiome (Brook *et al.*, 1991; Daniluk *et al.*, 2006; Paster *et al.*, 2001).

Here we report the characterization of a strict anaerobic strain DSM 24645^T, originally designated *Lachnospiraceae* bacterium ACC2, and isolated from the subgingival

plaque obtained from a 25-year-old black American female.

The study protocol was approved by the Institutional Review Board of Northeastern University; informed consent was obtained from the subject. According to the 16S rRNA gene sequence phylogeny, strain DSM 24645^T belongs to the family *Lachnospiraceae* within the phylum *Firmicutes* (Rainey, 2009). The novel isolate was enriched on liquid basic anaerobic medium (BM) supplemented with Na-citrate (2.0 g l⁻¹) and L-cysteine-HCl as a reducing agent and isolated in pure culture on BM-agar under anaerobic atmosphere (2 % H₂, 1 % CO₂, 97 % N₂) (Sizova *et al.*, 2012).

Colony morphology assessment was performed on Wilkins–Chalgren (WC) blood agar, BM, TY (trypticase peptone-yeast extract) and TGY (trypticase peptone-glucose-yeast extract) agar medium. All media were supplemented with L-cysteine-HCl as a reducing agent (Sizova *et al.*, 2012). Cell morphology was observed with a Leica DMBL light microscope equipped with phase-contrast. For electron microscopy, cells were fixed consecutively in (a) 2.5 % glutaraldehyde, 2.5 % formaldehyde in 0.1 M sodium cacodylate buffer pH 7.2 and (b) 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer, then dehydrated through a graded series of ethanol and embedded in a Spurr/Quetol epoxy resin mix (Ellis, 2006). Thin sections were stained with uranyl acetate and lead citrate and observed with a JEOL JEM 1010 transmission electron microscope and Tecnai G2 F20 scanning transmission electron microscope equipped with high-angle annular dark field (HAADF) and energy-dispersive X-ray spectroscopy (EDX) detectors. The Gram reaction was determined using Difco Gram stain kit. Resistance to various antibiotics and bile was tested with AN-IDENT (Oxoid), Sensi-Disc (BBL) and Remel susceptibility test disks; zones less than 10 mm were considered indicative of resistance. Oxidase, catalase and anaerobic nitrate reduction activities were tested with Remel reagents. Biochemical reactions and carbon source utilization were assessed with API 20A tests, Biolog AN microplates and with cultures grown on BM when yeast extract and Casamino acids were substituted with individual carbon sources including sugars, organic acids, milk, trypticase peptone, and yeast extract or Casamino acids alone. Effect of pH and temperature was assessed with cultures grown on TY medium. All experiments were conducted under anaerobic conditions; growth was scored as visible turbidity. Fermentation products were determined in cultures grown on BM with glucose (5 g l⁻¹) and yeast extract (0.5 g l⁻¹) by GC-MS (Agilent 6890 GC with a 5973 mass-selective detector; Agilent HP-5 capillary column; linear temperature gradient 110–260°C, helium as a carrier gas) and HPLC (Shimadzu VP series with UV-detector; ZORBAX Eclipse Plus C18 column with 5 µM H₂SO₄ as mobile phase). Cell biomass grown in TGY for 24 h was used for the whole-cell fatty acid and peptidoglycan analyses. Fatty acids were methylated, extracted and analysed by GC using the Sherlock Microbial Identification System at Microbial

ID, Inc. The peptidoglycan structure was analysed in the hydrolysates (4 M HCl, 100 °C, 16 h) by TLC on cellulose plates (Schumann, 2011). DNA base content (mol% G + C) was determined by HPLC (Cashion *et al.*, 1977; Mesbah *et al.*, 1989; Tamaoka & Komagata, 1984). Both analyses were performed at the Identification Service of the German Collection of Microorganisms and Cell Cultures (DSMZ). The 16S rRNA gene sequence was compared with the gene sequences available from GenBank; phylogenetic analysis was performed as described previously (Sizova *et al.*, 2012). Whole-genome sequencing was carried out by the Broad Institute of Harvard and MIT as part of the ‘*Lachnospiraceae* bacterium Group Sequencing Project’. The draft genome sequence of strain DSM 24645^T is available at <http://www.broadinstitute.org/> as *Lachnospiraceae* bacterium ACC2. The genome sequence of strain DSM 24645^T was compared with available genome sequences of other members of the family *Lachnospiraceae*. Individual coding sequences were submitted to the Rapid Annotation Subsystem Technology (RAST) server (Aziz *et al.*, 2008) for subsystem annotations.

Cells were non-spore-forming non-motile rods 4–40 µm long and 0.5–0.8 µm wide, often swollen, occurring singly or in chains (Fig. 1, Fig. S1, Table 1). About 10 % of cells grown on BM, TY and TGY media were represented by curved filaments up to 200 µm long. More than 50 % of cells grown on citrate and maltose media as well as at elevated pH or temperature formed long, curved filaments. Cells appeared Gram-variable after staining, but were structurally Gram-positive (Fig. 1c). Additionally, seven RAST-annotated genes associated with synthesis of teichoic and lipoteichoic acids were present in the genome (Table 2, S1). After 48–72 h incubation on WC blood agar plates, colonies were 2–3 mm in diameter and umbonate with irregular wavy edges. Colonies were non-pigmented and non-haemolytic. On BM-agar colonies appeared black. A diffusible black pigment was observed on BM and TY liquid media after 2–3 days incubation. The pigment was visible as a grainy substance surrounding cells (Fig. 1a). Most cells contained numerous intracellular nanometre-sized particles with a mean size of approximately 40 nm (Fig. 1c). EDX analysis (Müller & Engel, 2006; Sousa *et al.*, 2008) revealed that the particles were rich in iron and sulfur with Fe to S ratio about 1 : 1, and thus likely ferrous sulfide (FeS). Electron diffraction patterns showed diffuse rings, typical for poorly crystalline or completely amorphous nanoparticles. This is in stark contrast to the well-ordered iron sulfide crystals in magnetotactic bacteria (Pósfai *et al.*, 1998). Upon inspection of the genome, we identified a gene putatively annotated as encoding the cysteine desulfurase enzyme (EC 2.8.1.7), whose activity likely explains the production of FeS precipitate (Frazzon & Dean, 2003; Mihara & Esaki, 2002). Similar amorphous particles were previously observed in the cells of some dissimilatory sulfate-reducing bacteria grown with high concentrations of iron (Jones *et al.*, 1976). To our knowledge, this is the first report of such particles in a non-dissimilatory sulfate-reducing bacterium.

Fig. 1. Transmission electron micrographs of ultrathin sections of strain DSM 24645^T cells show their general morphology when grown on BM (a) and TY (b, c) media. Extracellular ferrous sulfide precipitate produced by cysteine desulfurase (a), and Gram-positive cell structure and intracellular nanometre-sized iron- and sulfur-rich particles (c) are also shown. Bars, 2 µm (a), 500 nm (b) and 100 nm (c).

Strain DSM 24645^T grew only under strict anaerobic conditions. Growth occurred at 30–42 °C, with optimum at 37 °C, and pH ranging from 4.9 to 7.5. The isolate was susceptible to discs containing 1 mg kanamycin, 10 µg colistin, 5 µg vancomycin, 50 µg metronidazole, 2 U penicillin, 15 mg rifampicin, 60 µg erythromycin, 10 µg ampicillin, 30 µg tetracycline and bile; and resistant to 25 µg sulfamethoxazole trimethoprim. Catalase, oxidase and urease activities were negative; nitrate and nitrite reduction was not detected. Gelatin was not liquefied, aesculin was not hydrolysed, indole was not produced, and milk was not coagulated but used for growth as carbon and energy source. Acid was produced on API 20A media containing glucose, maltose and sucrose, but not arabinose, cellobiose, lactose, mannose, melezitose, raffinose, rhamnose, trehalose, xylose, glycerol, mannitol, salicin or sorbitol. On Biolog AN media, the strain was able to utilize glucose, fructose, galactose, maltose, raffinose, sucrose, dextrin, fumaric acid, α -ketobutyric acid, malic acid, citric acid, pyruvic acid, alanyl-glutamine, asparagine, glutamic acid, glutamine and serine. Yeast extract was required for growth on glucose, citrate and pyruvate liquid media. The major metabolic end products of glucose fermentation were butyric, lactic, isovaleric and acetic acids.

The whole-cell hydrolysate contained *meso*-diaminopimelic acid (*meso*-DAP), indicating peptidoglycan type A1 γ (or A1 γ' ; A31 or A32.1) according to Schleifer & Kandler (1972) and Schumann (2011). Twelve RAST-annotated genes associated with diaminopimelic acid (DAP) synthesis were present in the genome (Table 2). The G + C content of DNA was 55.3 mol%, as determined by HPLC, and 55.05 mol% as calculated from the whole-genome sequence. The fatty acid profile showed that the strain contained C_{14:0} (39.9%), C_{14:0} DMA (dimethyl aldehyde) (7.7%), C_{16:0} (8.9%), C_{16:1} ω 7c DMA (15.2%) as major fatty acids; minor amounts of C_{16:1} ω 7c (5.7%), C_{16:0} DMA (5.2%) and trace amounts of C_{12:0} (1.7%), C_{11:0} DMA (0.3%), C_{14:11} ω 7c DMA (0.9%), iso-C_{15:0} (0.3%), anteiso-C_{15:0} (0.7%), C_{16:0} aldehyde (1.3%), C_{16:1} ω 5c (0.5%), C_{18:0} (0.3%), C_{18:1} ω 9c DMA (0.2%), C_{18:1} ω 7c DMA (1.0%) and C_{18:0} DMA (0.2%). In the whole genome there were no predicted gene sequences found with recognizable similarity to those responsible for respiratory lipoquinones, mycolic acids and lipopolysaccharides biosynthesis. At the same time 13 and 10 RAST-annotated genes associated with polar lipids and polyamines synthesis, respectively, were present in the genome (Table 2).

The 16S rRNA gene-based phylogenetic tree showed that strain DSM 24645^T, together with several uncultured oral clones, formed a separate branch within the family *Lachnospiraceae* (Fig. 2). The nearest phylogenetic relative of the strain DSM 24645^T was *M. indoligenes* (Carlier *et al.*, 2007), sharing 94.2% of 16S rRNA gene sequence similarity, which was below a 'lower cut-off window' of 95% for the new genus differentiation (Yarza *et al.*, 2008; Tindall *et al.*, 2010). The family *Lachnospiraceae* comprises other micro-organisms isolated from the human oral cavity that represent genera *Oribacterium*, *Shuttleworthia*, *Catonella*

Table 1. Characteristics that differentiate strain DSM 24645^T from phylogenetically related organisms within the family *Lachnospiraceae* isolated from the human body

Taxa: 1, DSM 24645^T (data from this study); 2, *M. indoligenes* (Carlier *et al.*, 2007); 3, *O. sinus* (Carlier *et al.*, 2004); 4, *S. satelles* (Downes *et al.*, 2002); 5, *C. morbi* (Moore & Moore, 1994); 6, *J. ignava* (Moore & Moore, 1994); 7, *R. intestinalis* (Duncan *et al.*, 2002); 8, *D. longicatena* (Taras *et al.*, 2002); 9, *R. peoriensis* (Cotta *et al.*, 2009). All organisms were isolated from human sources. ND, No data available.

Characteristic	1	2	3	4	5	6	7	8	9
Cell shape	Large rods, often swollen	Elongated rod with pointed ends	Elongated ovoid rods	Slightly curved rods	Rods with central swelling	Rods	Slightly curved rods	Rods	Short oval to rod cells
Cell size (µm)	4–40 × 0.5–0.8	0.8–1.7 × 0.5–0.6	1.7–2.2 × 0.8–1.0	0.4–0.6 × 1.0–2.5	0.7–1.2 × 1.6–4.0	0.8 × 3.7–6.4	0.4–0.5 × 1.0–5.0	0.6–1.4 × 0.8–4.7	ND
Motility/flagella	–	–	+ (laterally inserted)	–	–	–	+ (subterminal)	–	–
Spore formation	–	–	–	–	–	–	–	–	+
Gram-stain	Variable	Variable	Negative	Positive	Negative	Negative	Positive	Positive	Positive
Cell-wall structure	Gram-positive	Gram-positive	Gram-positive	ND	ND	ND	ND	ND	ND
Acid from:									
Lactose	–	–	–	Certain strains	+	–	–	+	+
Maltose	+	–	–	+	+	–	+	+	+
Oxygen requirements	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic	Anaerobic
Growth:									
Temperature range	30–42	37	37	30–37	37	37	30–45	30–45	37
pH range	4.9–7.5	ND	ND	ND	ND	ND	ND	7	ND
Metabolic end-products*	B, L, iV, A	A, B, L	A, L	A, B, L	A, F, L	A, iV	B, F, L, H ₂ , CO ₂	A, F, E	A, S
Indole formation	–	+	+	+	–	–	–	–	–
DNA G+C content (mol%)	55.05–55.3	50.2	42.4	51	34	32	29–31	43.8–45.6	48.7
Major FAMES	C _{14:0} , C _{14:0} DMA, C _{16:0} , C _{16:1} ω7c, DMA	iso-C _{13:0} 2-OH, C _{14:0} , C _{16:0}	C _{14:0} , anteiso-C _{15:0} , C _{15:0}	ND	C _{14:0} , C _{14:0} DMA, C _{16:0}	C _{14:0} , C _{16:0}	ND	ND	C _{16:0} , C _{16:1} ω7c, C _{18:1} ω7c
16S rRNA similarity with DSM 24645 ^T (%)	100	94.2	89.3	89.2	87	86	90.9	90.9	89.7
Isolation source	Subgingival plaque	Buttock, thigh and intra-abdominal abscess	Maxillary sinus	Subgingival plaque	Gingival crevice	Gingival crevice	Faeces	Faeces	Wound

*A, acetate; B, butyrate; L, lactate; F, formate; E, ethanol; S, succinate; iV, iso-valerate.

Table 2. Number of genes identified in biosynthetic pathway from whole genome sequences of strain DSM 24645^T and related organisms identified by RAST

Taxa: 1, DSM 24645^T; 2, *Oribacterium sinus* strain F0268; 3, *Shuttleworthia satelles*; 4, *Catonella morbi*; 5, *Roseburia intestinalis*; 6, *Dorea longicatena*. Data are for type strains except where indicated otherwise. Numbers of genes identified for benzoquinones (ubiquinones, rhodoquinones, plastoquinones), naphthoquinones (menaquinones, demethylmenaquinones, monomethylmenaquinones, menathioquinones) and mycolic acids were zero for all taxa studied.

Genes responsible for biosynthesis	1	2	3	4	5	6
Accession number	AGEL01000000	PRJNA34635	PRJNA33169	PRJNA33121	PRJNA30005	PRJNA18157
Teichoic and lipoteichoic acids	7	4	4	13	13	0
Polar lipids	13	20	19	17	29	32
Lipopolysaccharides	0	0	0	4	0	0
Polyamines	10	8	4	7	15	11
Diaminopimelic acid	12	10	6	0	10	14

and *Johnsonella* (Carlier *et al.*, 2004; Downes *et al.*, 2002; Moore & Moore, 1994; Rainey, 2009; Willems & Collins, 1995). Each genus contains a single species, *Oribacterium sinus*, *Shuttleworthia satelles*, *Catonella morbi* and *Johnsonella ignava*, showing 89.3, 89.2, 87.4 and 86.7 % 16S rRNA gene sequence similarity with strain DSM 24645^T, respectively.

Analysis of the genome sequences of *O. sinus*, *S. satelles*, *C. morbi*, *Roseburia intestinalis* and *Dorea longicatena* available from NCBI revealed different number of RAST-annotated genes associated with synthesis of teichoic and lipoteichoic acids and DAP in all genomes except *D. longicatena* and *C. morbi*, respectively. Genes associated with synthesis of

Fig. 2. Minimum-evolution phylogenetic tree based on 16S rRNA gene sequence comparisons of the strain DSM 24645^T and other members of the family Lachnospiraceae. A 1491 bp long 16S rRNA sequence of the strain DSM 24645^T was retrieved from the complete genome sequence. Bootstrap values >50% calculated for 1000 subsets are shown at branch-points. Bar, 0.02 substitutions per position.

polar lipids and polyamines were present in all tested genomes, but not genes responsible for respiratory lipoquinones and mycolic acids biosynthesis. Genes associated with synthesis of lipopolysaccharides were found in *C. morbi* only (Table 2).

All available data on strain DSM 24645^T and related members of the *Lachnospiraceae* family, isolated from the human body, are summarized in Table 1 and 2. Three strains of *M. indoligenes*, the closest 16S phylogenetic relatives of strain DSM 24645^T were isolated from the human buttock, thigh and intra-abdominal abscesses, whereas strain DSM 24645^T was isolated from a non-infectious subgingival plaque sampled from a systemically and dentally healthy human subject. *M. indoligenes* cells were 0.8–1.7 µm long rods, compared to cells 4–40 µm and up to 200 µm long for strain DSM 24645^T. In contrast to strain DSM 24645^T, *M. indoligenes* produced indole, as did two other oral bacteria, *S. satelles* and *O. sinus*. Strain DSM 24645^T exhibited numerous nanometre-sized particles rich in iron and sulfur not reported in other members of the *Lachnospiraceae* family. DNA G + C content, FAME profile, peptidoglycan type, utilized carbon sources, and metabolic end products as well as the number of annotated genes responsible for biosynthesis of teichoic and lipoteichoic acids, polar lipids, polyamines and DAP, differentiate strain DSM 24645^T substantially from *M. indoligenes* and other related members of the *Lachnospiraceae* family. On the basis of physiological, biochemical and molecular properties, we propose that the strictly anaerobic strain DSM 24645^T represents a new genus and species for which we propose the name *Stomatobaculum longum* gen. nov., sp. nov.

Description of *Stomatobaculum* gen. nov.

Stomatobaculum (Sto.ma.to.ba'cu.lum. Gr. n. *stoma* -atos mouth; L. neut. n. *baculum* stick, rod; N.L. neut. n. *Stomatobaculum* mouth rod, because the organism was first isolated from the human mouth).

Cells are Gram-variable after staining but structurally Gram-positive, long non-motile rods, about 4–40 µm long and 0.5–0.8 µm wide, often swollen, occurring singly, in chains or as curved filaments up to 200 µm long. No spores are formed. Strictly anaerobic. Catalase, oxidase and urease are negative. Nitrate is not reduced. Growth is supported by yeast extract, trypticase peptone, milk, citrate, glucose and maltose but not Casamino acids or starch. Yeast extract is required for growth on sugars and organic acids. The major metabolic end-products of glucose fermentation are butyrate, lactate, isovalerate and acetate. Growth temperature range is 30–42 °C and the pH range is 4.9–7.5. Major fatty acids are C_{14:0} (39.9%), C_{14:0} DMA (dimethyl aldehyde) (7.7%), C_{16:0} (8.9%), C_{16:1ω7c} DMA (15.2%). The peptidoglycan type is A1γ (or A1γ'; A31 or A32.1). Genes responsible for biosynthesis of teichoic and lipoteichoic acids, polar lipids, polyamines and DAP are present in the genome. There are no genes responsible for

biosynthesis of respiratory lipoquinones, mycolic acids or lipopolysaccharides. The type species is *Stomatobaculum longum*. Comparative analysis of the 16S rRNA gene sequence shows that the genus *Stomatobaculum* represents a separate line of descent within the family *Lachnospiraceae*.

Description of *Stomatobaculum longum* sp. nov.

Stomatobaculum longum [lon'gum. L. neut. adj. *longum* long (in shape)].

Cell morphology is as described for the genus. Colonies are umbonate with irregular wavy edges, 2–3 mm in diameter, non-haemolytic, non-pigmented on WC agar and black-pigmented on BM-agar. Black pigment is produced in TY or TGY liquid medium. Bacterial cells accumulate numerous nanometre-sized iron- and sulfur-rich particles, distributed randomly within cells. Acid is produced from glucose, maltose and sucrose, but not from arabinose, cellobiose, lactose, mannose, melezitose, raffinose, rhamnose, trehalose, xylose, glycerol, mannitol, salicin or sorbitol. Indole is not produced. Gelatin is not liquefied. Aesculin is not hydrolysed. The type strain is susceptible to kanamycin, colistin, vancomycin, metronidazole, penicillin, rifampicin, erythromycin, ampicillin, tetracycline and bile but is resistant to sulfamethoxazole trimethoprim. DNA G + C content is 55.05–55.3 mol%.

The type strain is DSM 24645^T (=HM-480^T; deposited in BEI Resources, an NIH collection managed by the ATCC), isolated from human subgingival dental plaque.

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