Non contiguous-finished genome sequence and description of *Enterobacter massiliensis* sp. nov.

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Enterobacter massiliensis strain JC163^T sp. nov. is the type strain of *E. massiliensis* sp. nov., a new species within the genus *Enterobacter*. This strain, whose genome is described here, was isolated from the fecal flora of a healthy Senegalese patient. *E. massiliensis* is an aerobic rod. Here we describe the features of this organism, together with the complete genome sequence and annotation. The 4,922,247 bp long genome (1 chromosome but no plasmid) exhibits a G+C content of 55.1% and contains 4,644 protein-coding and 80 RNA genes, including 5 rRNA genes.

Introduction

Enterobacter massiliensis strain JC163^T (= CSUR P161 = DSM 26120) is the type strain of *E. massiliensis* sp. nov. This bacterium is a Gram-negative, aerobic, flagellate, indole-positive bacillus that was isolated from the feces of a healthy Senegalese patient in a study aiming at cultivating all bacterial species in human feces [1]. The current classification of prokaryotes, known as polyphasic taxonomy, relies on a combination of phenotypic and genotypic characteristics [2]. However, as more than 3,000 bacterial genomes have been sequenced [3] and the cost of genomic sequencing is decreasing, we recently proposed to integrate genomic information in the description of new bacterial species [4-15].

Here we present a summary classification and a set of features for *E. massiliensis* sp. nov. strain JC163^T (= CSUR P161 = DSM 26120), together with the description of the complete genomic sequencing and annotation. These characteristics support the circumscription of the species *E. massiliensis.* The genus *Enterobacter* (Hormaeche and Edwards, 1960) was created in 1960 [16]. To date, this genus is comprised of 25 species [17-35] and 2 subspecies. Members of the genus were isolated mostly from the environment, in particular from plants and fruits, but are also frequently isolated from humans, notably in health-care associated infections, causing bacteremia, pneumonia or urinary tract infections [36]. In addition, many *Enterobacter* spp. were isolated from the normal fecal flora.

Classification and features

A stool sample was collected from a healthy 16-year-old male Senegalese volunteer patient living in Dielmo (rural village in the Guinean-Sudanian zone in Senegal), who was included in a research protocol. Written assent was obtained from this individual. No written consent was needed from his guardians for this study because he was older than 15 years old (in accordance with the previous project approved by the Ministry of Health of Senegal and the assembled village population and as published elsewhere [37]).

Both this study and the assent procedure were approved by the National Ethics Committee of Senegal (CNERS) and the Ethics Committee of the Institut Fédératif de Recherche IFR48, Faculty of Medicine, Marseille, France (agreement numbers 09-022 and 11-017). Several other new bacterial species were isolated from this specimen using various culture conditions, including the recently described *Alistipes senegalensis*, *Alistipes timonensis*, *Anaerococcus senegalensis*, *Bacillus timonensis*, *Clostridium senegalense*, *Peptoniphilus timonensis*, *Paenibacillus senegalensis*, *Herbaspirillum massiliense*, *Kurthia massiliensis*,

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Brevibacterium *senegalense*, *Aeromicrobium* *massiliense* and *Cellulomonas* *massiliensis* [4-15].

The fecal specimen was preserved at -80°C after collection and sent to Marseille. Strain JC163[T] (Table 1) was isolated in April 2011 by aerobic cultivation on Brain-Heart Infusion (BHI) agar at 37°C after preincubation of the stool specimen with lytic *E. coli* T1 and T4 phages [1,50]. This strain exhibited a nucleotide sequence similarity with *Enterobacter* species ranging from 95.74% with *E. pyrinus* (Chung *et al*., 1993) to 97.33% with *E. cloacae* subsp. *cloacae* (Jordan, 1980) (Figure 1). This latter value was lower than the 98.7% 16S rRNA gene sequence threshold recommended by Stackebrandt and Ebers to delineate a new species without carrying out DNA-DNA hybridization [51].

Table 1. Classification and general features of *Enterobacter massiliensis* strain JC163[T]

MIGS ID	Property	Term	Evidence code[a]
		Domain: *Bacteria*	TAS [38]
		Phylum *Proteobacteria*	TAS [39]
		Class *Gammaproteobacteria*	TAS [40,41]
	Current classification	Order "*Enterobacteriales*"	TAS [42]
		Family *Enterobacteriaceae*	TAS [43-45]
		Genus *Enterobacter*	TAS [16,44,46-48]
		Species *Enterobacter massiliensis*	IDA
		Type strain: JC163[T]	IDA
	Gram stain	Negative	IDA
	Cell shape	Rod	IDA
	Motility	Motile	IDA
	Sporulation	Nonsporulating	IDA
	Temperature range	Mesophile	IDA
	Optimum temperature	37°C	IDA
MIGS-6.3	Salinity	Unknown	IDA
MIGS-22	Oxygen requirement	Aerobic	IDA
	Carbon source	Unknown	NAS
	Energy source	Unknown	NAS
MIGS-6	Habitat	Human gut	IDA
MIGS-15	Biotic relationship	Free living	IDA
	Pathogenicity	Unknown	
	Biosafety level	2	
MIGS-14	Isolation	Human feces	
MIGS-4	Geographic location	Senegal	IDA
MIGS-5	Sample collection time	September 2010	IDA
MIGS-4.1	Latitude	13.7167	IDA
MIGS-4.1	Longitude	– 16.4167	IDA
MIGS-4.3	Depth	Surface	IDA
MIGS-4.4	Altitude	51 m above sea level	IDA

Evidence codes - IDA: Inferred from Direct Assay; TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project [49]. If the evidence is IDA, then the property was directly observed for a live isolate by one of the authors or an expert mentioned in the acknowledgements.

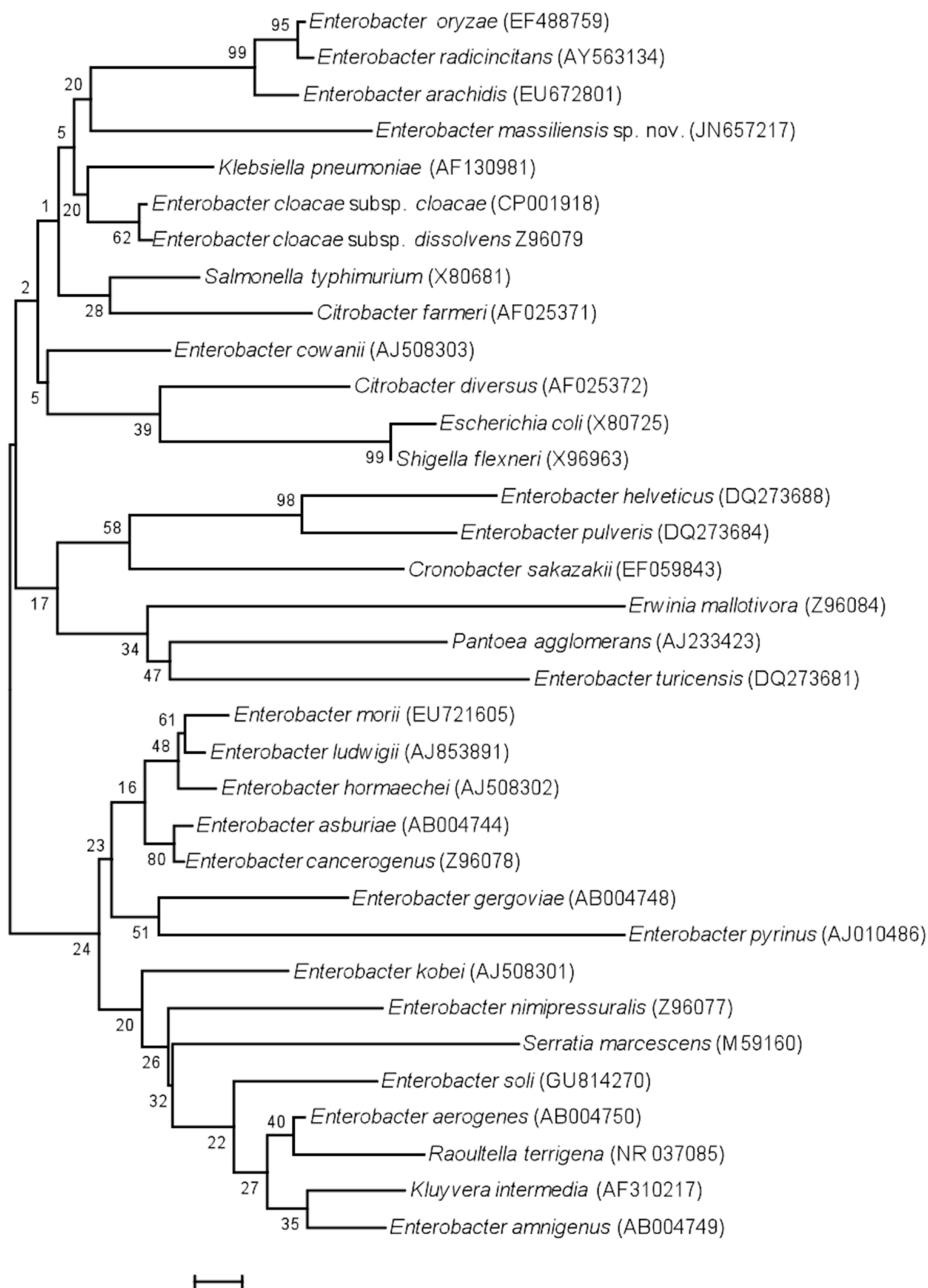

Figure 1. Phylogenetic tree highlighting the position of *Enterobacter massiliensis* strain JC163 relative to other type strains within the *Enterobacter* genus. Members of phylogenetically closely related genera were also included. GenBank accession numbers are indicated in parentheses. Sequences were aligned using CLUSTALW, and phylogenetic inferences obtained using the maximum-likelihood method within the MEGA software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 500 times to generate a majority consensus tree. The scale bar represents a 0.2% nucleotide sequence divergence.

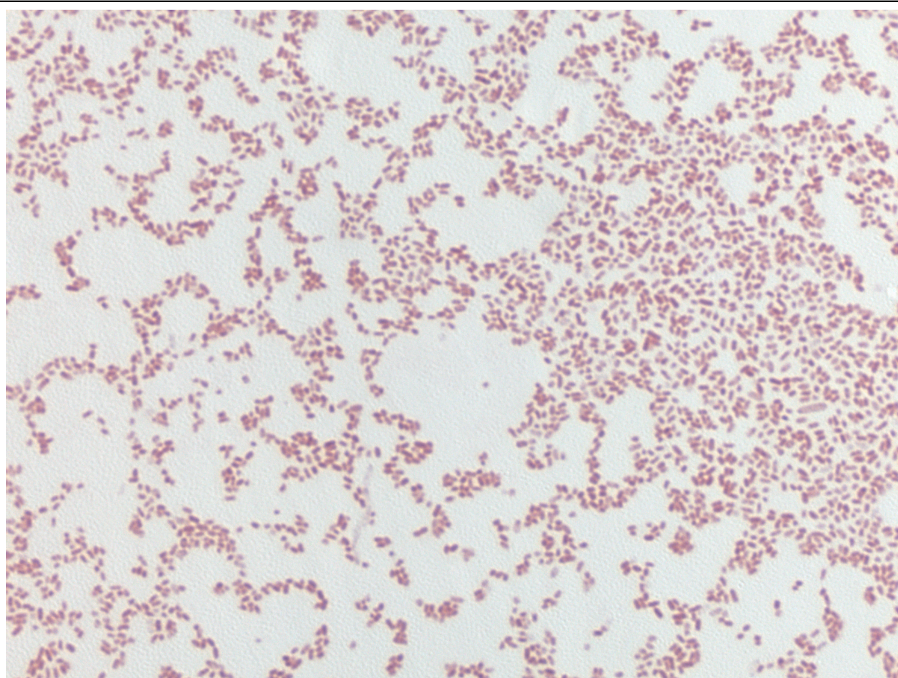

Figure 2. Gram staining of *E. massiliensis* strain JC163^T

Different growth temperatures (25, 30, 37, 45°C) were tested; growth occurred between 25 and 45°C, optimal growth was observed between 30 and 37°C. Colonies were convex, opaque, light-cream colored and circular with regular margins and with a diameter of 2 mm on BHI agar. Growth of the strain was tested under anaerobic and microaerophilic conditions using GENbag anaer and GENbag microaer systems, respectively (BioMerieux), and under aerobic conditions, with or without 5% CO_2. Optimal growth was obtained under aerobic conditions in the presence of 5% CO_2. Weak growth was observed under microaerophilic condition. No growth was obtained anaerobically. A motility test was positive. Cells grown on agar are Gram-negative rods (Figure 2) and have a mean diameter of 1.02 µm and a mean length of 1.90 µm and have several polar flagella (Figure 3).

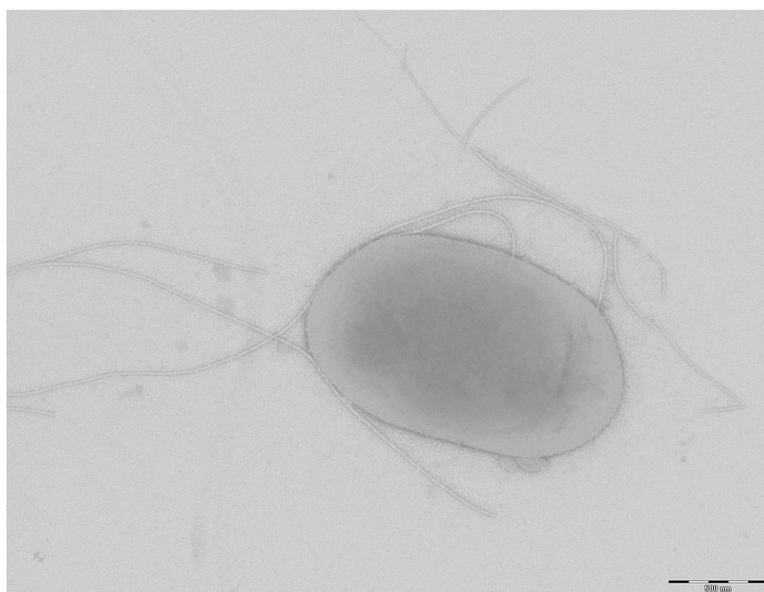

Figure 3. Transmission electron microscopy of *E. massiliensis* strain JC163^T, using a Morgani 268D (Philips) at an operating voltage of 60kV. The scale bar represents 500 nm.

Strain JC163^T exhibited catalase activity but not oxidase activity. Using the API 20E system, positive reactions were obtained for indole production, β-galactosidase and glucose, mannitol, sorbitol and rhamnose fermentation. *E. massiliensis* is susceptible to ticarcillin, imipenem, trimethoprim/sulfamthoxazole, gentamicin, amikacin, and colimycin but resistant to fosfomycin and nitrofurantoin. By comparison with *E. arachidis*, its phylogenetically-closest neighbor, *E. massiliensis* differed in arginine dihydrolase, ornithine decarboxylase, citrate and succinate fermentation [19].

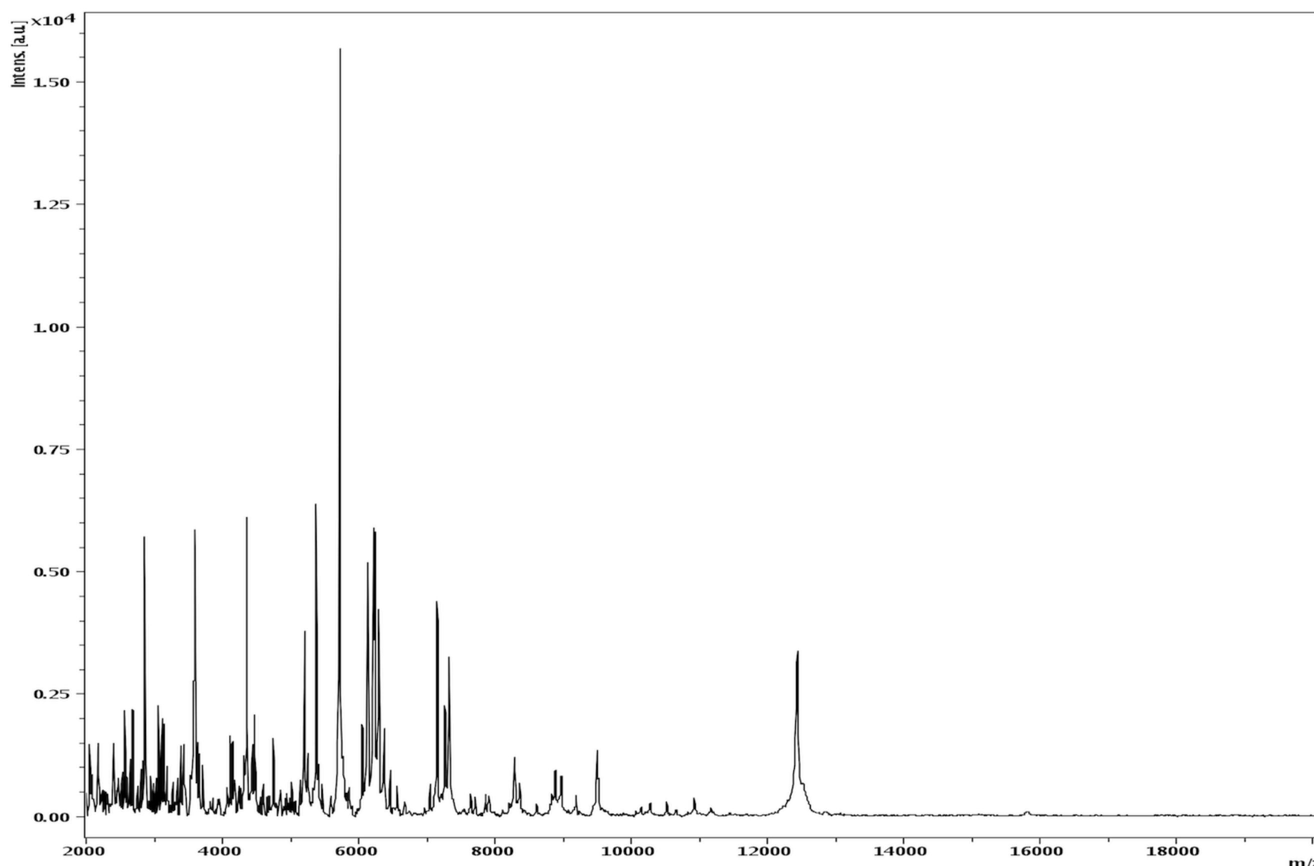

Figure 4. Reference mass spectrum from *E. massiliensis* strain JC163T. Spectra from 12 individual colonies were compared and a reference spectrum was generated.

Matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) MS protein analysis was carried out as previously described [52]. Briefly, a pipette tip was used to pick one isolated bacterial colony from a culture agar plate and spread it as a thin film on a MTP 384 MALDI-TOF target plate (Bruker Daltonics, Germany). Twelve distinct deposits were done for strain JC163^T from twelve isolated colonies. Each smear was overlaid with 2 µL of matrix solution (saturated solution of alpha-cyano-4-hydroxycinnamic acid) in 50% acetonitrile, 2.5% tri-fluoracetic acid, and allowed to dry for five minutes. Measurements were performed with a Microflex spectrometer (Bruker). Spectra were recorded in the positive linear mode for the mass range of 2,000 to 20,000 Da (parameter settings: ion source 1 (ISI), 20kV; IS2, 18.5 kV; lens, 7 kV). A spectrum was obtained after 675 shots at a variable laser power. The time of acquisition was between 30 seconds and 1 minute per spot. The twelve JC163^T spectra were imported into the MALDI BioTyper software (version 2.0, Bruker) and analyzed by standard pattern matching (with default parameter settings) against the main spectra of 3,769 bacteria, including spectra from 34 spectra from validly published *Enterobacter* species that were used as reference data in the BioTyper database (updated March 15th, 2012). The method of identification includes the m/z from 3,000 to 15,000 Da. For every spectrum, a maximum of 100

peaks were taken into account and compared with the spectra in database. A score enabled the presumptive identification and discrimination of the tested species from those in a database: a score $\geq$ 2 with a validly published species enabled the identification at the species level; a score $\geq$ 1.7 but < 2 enabled the identification at the genus level; and a score < 1.7 did not enable any identification. For strain JC163^T, the score obtained was 1.4, suggesting that our isolate was not a member of a known species. We incremented our database with the spectrum from strain JC163^T (Figure 4). In addition, the gel view allows the highlighting of spectra differences with other of *Enterobacteriaceae* family members (Figure 5).

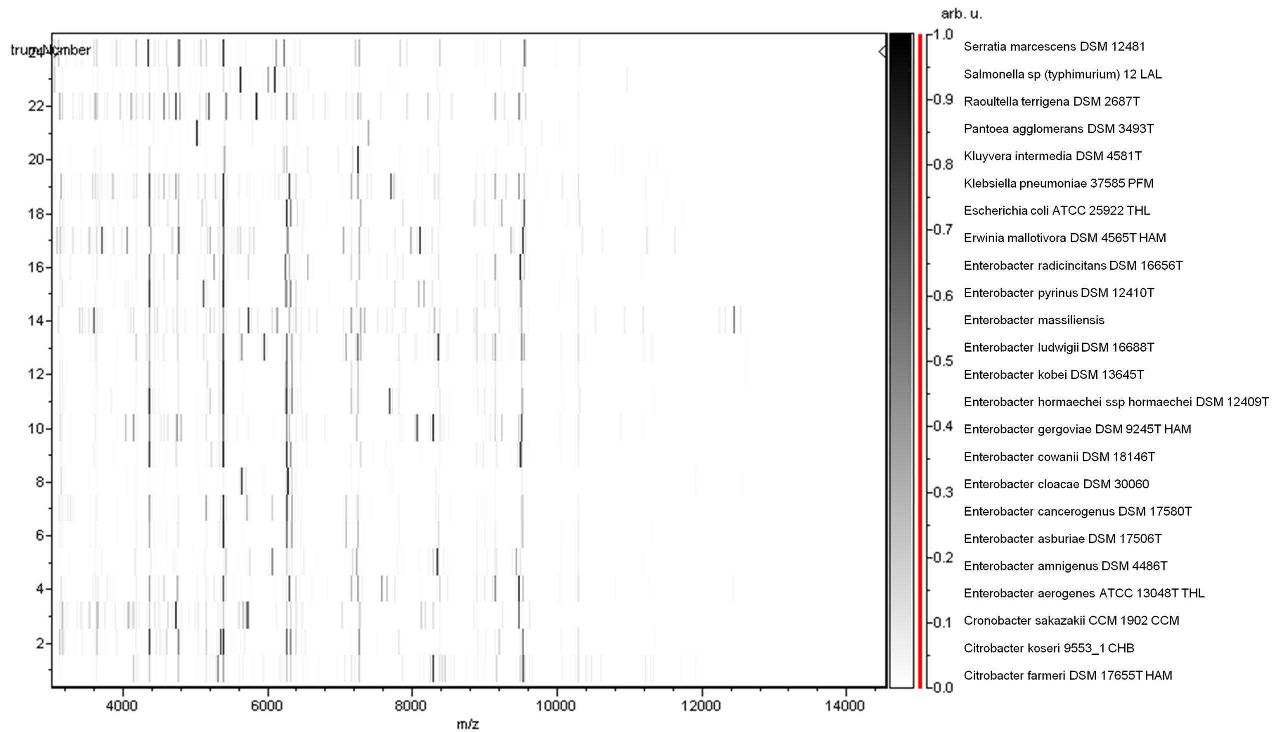

Figure 5. Gel view comparing *Enterobacter massiliensis* JC163^T spectra with 23 other members into *Enterobacteriaceae* family. The Gel View displays the raw spectra of all loaded spectrum files arranged in a pseudo-gel like look. The x-axis records the m/z value. The left y-axis displays the running spectrum number originating from subsequent spectra loading. The peak intensity is expressed by a Gray scale scheme code. The color bar and the right y-axis indicate the relation between the color a peak is displayed with and the peak intensity in arbitrary units.

Genome sequencing information

Genome project history

The organism was selected for sequencing on the basis of its phylogenetic position and 16S rRNA similarity to other members of the genus *Enterobacter*, and is part of a study of the human digestive flora aiming at isolating all bacterial species within human feces. It was the 10th genome of an *Enterobacter* species (including genomes from 5 validly published species) and the first genome of *E. massiliensis* sp. nov. The genome sequence deposited in GenBank under accession number CAEO00000000 consists of 224 contigs. Table 2 shows the project information and its association with MIGS version 2.0 compliance [53].

Growth conditions and DNA isolation

E. massiliensis strain JC163^T, (= CSUR P161 = DSM 26120) was grown aerobically on BHI agar at 37°C. Four petri dishes were spread and resuspended in 3×100µl of G2 buffer. A first mechanical lysis was performed by glass powder on the Fastprep-24 device (Sample Preparation system, MP Biomedicals, USA) during 2×20 seconds. DNA was then treated with 2.5 µg/µL lysozyme (30 minutes at 37°C) and extracted through the BioRobot EZ 1 Advanced XL (Qiagen). The DNA was then concentrated and purified on a Qiamp kit (Qiagen). The yield and the concentration was measured by the Quant-it Picogreen kit (Invitrogen) on the Genios_Tecan fluorometer at 118 ng/µl.

Table 2. Project information

MIGS ID	Property	Term
MIGS-31	Finishing quality	High-quality draft
MIGS-28	Libraries used	Paired-end 3 Kb library
MIGS-29	Sequencing platforms	454 GS FLX Titanium
MIGS-31.2	Fold coverage	13×
MIGS-30	Assemblers	Newbler version 2.5.3
MIGS-32	Gene calling method	Prodigal
	EMBL ID	CAEO00000000
	EMBL Date of Release	November 19, 2012
MIGS-13	Project relevance	Study of the human gut microbiome

Genome sequencing and assembly

A 3kb paired-end sequencing strategy (Roche, Meylan, France) was used. Five µg of DNA was mechanically fragmented on the Hydroshear device (Digilab, Holliston, MA, USA) with an enrichment size at 3-4kb. The DNA fragmentation was visualized using an Agilent 2100 BioAnalyzer on a DNA labchip 7500, with an optimal size of 4.648kb.The library was constructed according to the 454_Titanium paired end protocol (Roche). Circularization and nebulization were performed and generated a pattern with an optimal at 437 bp. Following PCR amplification through 15 cycles followed by double size selection, the single stranded paired-end library was then quantified on the Quant-it Ribogreen kit (Invitrogen) on the Genios_Tecan fluorometer at 122pg/µL. The library concentration equivalence was calculated as 5.12E+08 molecules/µL. The library was stored at -20°C until use. The library was clonally amplified with 1 cpb in 4 emPCR reactions with the GS Titanium SV emPCR Kit (Lib-L) v2 (Roche). The yield of the emPCR was 14.45%, in the 5 to 20% range recommended by the Roche procedure.

Approximately 790,000 beads were loaded on ¼ region of a GS Titanium PicoTiterPlate (PTP Kit 70x75, Roche) and pyrosequenced with the GS Titanium Sequencing Kit XLR70 and the GS FLX Titanium sequencer (Roche). The run was performed overnight and then analyzed on the cluster through the gsRunBrowser and Newbler assembler (Roche). A total of 283,817 passed filter wells generated 80.8 Mb with a length average of 284 bp. The passed filter sequences were assembled using Newbler with 90% identity and 40 bp as overlap. The final assembly identified 224 contigs arranged in 11 scaffolds and generated a genome size of 4.92 Mb.

Genome annotation

Open Reading Frames (ORFs) were predicted using Prodigal [54] with default parameters but the predicted ORFs were excluded if they spanned a sequencing gap region. The predicted bacterial protein sequences were searched against the GenBank database [55] and the Clusters of Orthologous Groups (COG) databases using BLASTP. The tRNAScanSE tool [56] was used to find tRNA genes, whereas ribosomal RNAs were found by using RNAmmer [57] and BLASTN against the GenBank database. Signal peptides and numbers of transmembrane helices were predicted using SignalP [58] and TMHMM [59] respectively.

To estimate the mean level of nucleotide sequence similarity at the genome level between *E. massiliensis* strain JC163^T, *E. aerogenes* strain KCTC 2190 (GenBank accession number CP002824), *E. asburiae* strain LF7a (CP003026), *E. cancerogenus* strain ATCC35316 (ABWM00000000), *E. cloacae* subsp. *cloacae* strain ATCC13047 (CP001918), *E. cloacae* subsp. *dissolvens* strain SDM (CP003678) and *E. hormaechei* strain ATCC49162 (AFHR00000000), we compared the ORFs only using BLASTN and the following parameters: a query coverage of > 70% and a minimum nucleotide length of 100 bp.

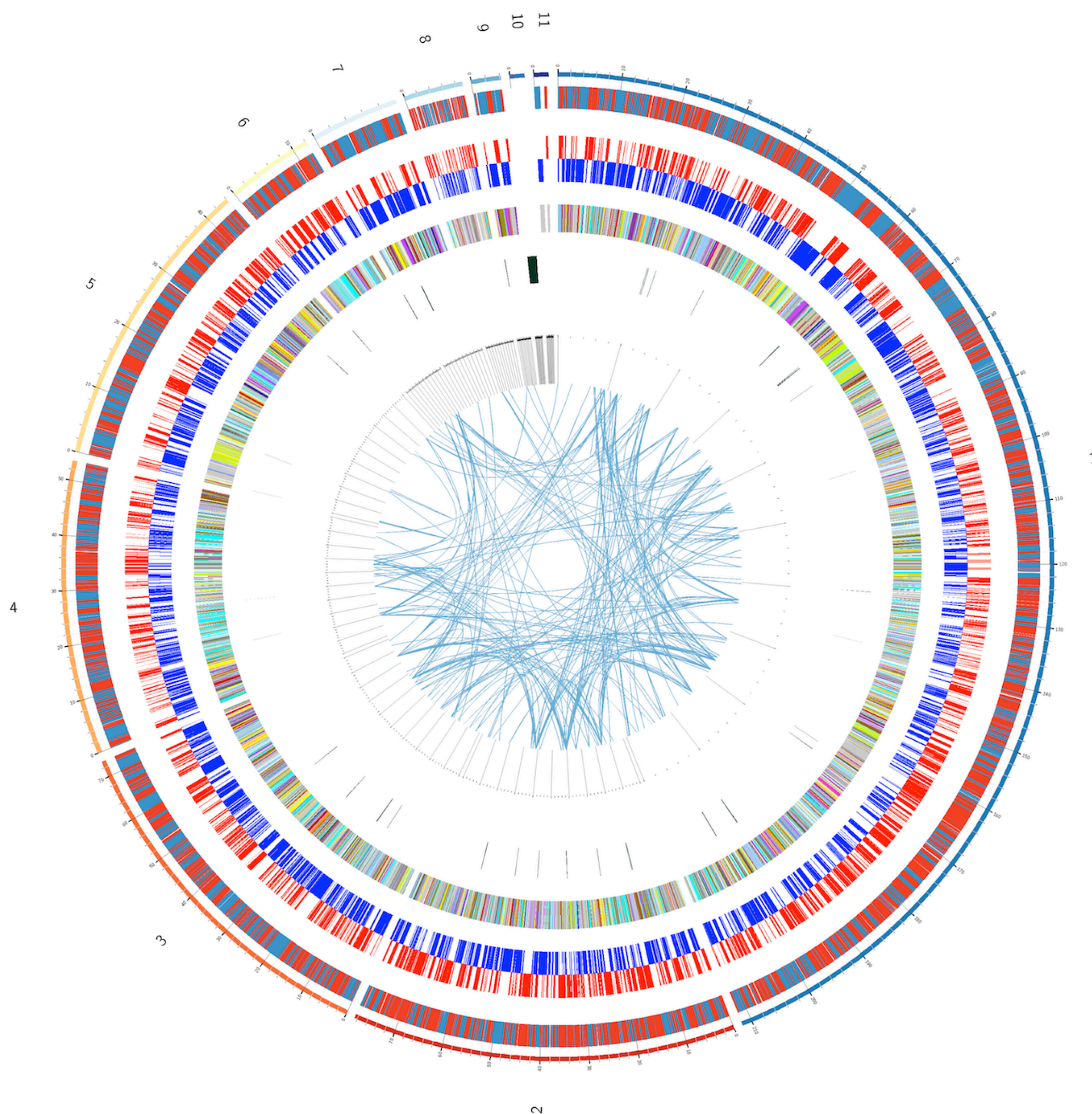

Figure 6. Graphical circular map of the chromosome. From outside to the center: genes on both the forward and reverse strands, genes on forward strand, genes on reverse strand, genes colored by COG categories, RNA genes (tRNAs and rRNAs) and blast of the genome *vs* itself.

Genome properties

The genome of *E. massiliensis* sp. nov. strain JC163^T is 4,922,247 bp long (1 chromosome but no plasmid) with a 55.1% G+C content (Figure 6 and Table 3). Of the 4,724 predicted genes, 4,644 were protein-coding genes, and 80 were RNAs, including 1 complete rRNA operon, 2 additional 5S rRNAs and 75 tRNAs. A total of 3,181 genes (68.5%) were assigned a putative function. The remaining genes were annotated as hypothetical or unknown proteins. The distribution of genes into COGs functional categories is presented in Table 4. The properties and the statistics of the genome are summarized in Tables 3 and 4.

Comparison with other *Enterobacter* species genomes

Here, we compared the genome of *E. massiliensis* strain JC163[T] with those of *E. aerogenes* strain KCTC 2190, *E. asburiae* strain LF7a, *E. cancerogenus* strain ATCC35316, *E. cloacae* subsp. *cloacae* strain ATCC13047, *E. cloacae* subsp. *dissolvens* strain SDM and *E. hormaechei* strain ATCC49162. The draft genome of *E. massiliensis* is smaller than those of *E. aerogenes*, *E. cloacae* subsp. *cloacae* and *E. cloacae* subsp. *dissolvens* (4.92, 5.28, 5.59 and 4.96Mb, respectively), but larger than those of *E. asburiae*, *E. cancerogenus* and *E. hormaechei* (3.81, 4.60 and 4.80, respectively). *E. massiliensis* has a similar G + C content to *E. cloacae* subsp. *dissolvens* (55.1%) but larger than *E. aerogenes*, *E. asburiae* and *E. cloacae* subsp. *cloacae* (54.8, 53.8 and 54.79%, respectively) and lower than *E. cancerogenus* and *E. hormaechei* (55.8 and 55.2%, respectively). *E. massiliensis* had a greater number of predicted genes than *E. cancerogenus* and *E. cloacae* subsp. *dissolvens* (4,724, 4,642 and 4,646, respectively), but a smaller number than *E. aerogenes*, *E. asburiae*, *E. cloacae* subsp. *cloacae* and *E. hormaechei* (5,021, 4,805, 5,627 and 4,779, respectively).

In addition, *E. massiliensis* shared a mean genome sequence similarity of 84.26% (range 70.05-100%), 83.89% (70.03-100%), 84.36% (70.05-100%), 84.14% (70.00-100%), 84.14% (70.05-100%) and 84.38% (70.24-100%) with *E. aerogenes*, *E. asburiae*, *E. cancerogenus*, *E. cloacae* subsp. *cloacae*, *E. cloacae* subsp. *dissolvens* and *E. hormaechei*, respectively.

Table 3. Nucleotide content and gene count levels of the genome

Attribute	Value	% of total[a]
Genome size (bp)	4,922,247	-
DNA coding region (bp)	4,284,197	87
DNA G+C content (bp)	2,712,016	55.1
Total genes	4,724	100
RNA genes	80	1.7
Protein-coding genes	4,644	98.3
Genes with function prediction	3,181	68.5
Genes assigned to COGs	3,710	80
Genes with peptide signals	372	8
Genes with transmembrane helices	702	15.1

[a]The total is based on either the size of the genome in base pairs or the total number of protein coding genes in the annotated genome.

Table 4. Number of genes associated with the 25 general COG functional categories

Code	Value	%age	Description
J	175	3.77	Translation
A	1	0.02	RNA processing and modification
K	387	8.33	Transcription
L	169	3.64	Replication, recombination and repair
B	0	0	Chromatin structure and dynamics
D	34	0.73	Cell cycle control, mitosis and meiosis
Y	0	0	Nuclear structure
V	46	0.99	Defense mechanisms
T	176	3.79	Signal transduction mechanisms
M	237	5.10	Cell wall/membrane biogenesis
N	143	3.08	Cell motility
Z	0	0	Cytoskeleton

Table 4 (cont.). Number of genes associated with the 25 general COG functional categories

Code	Value	%age	Description
W	0	0	Extracellular structures
U	143	3.08	Intracellular trafficking and secretion
O	138	2.97	Posttranslational modification, protein turnover, chaperones
C	218	4.69	Energy production and conversion
G	472	10.16	Carbohydrate transport and metabolism
E	463	9.97	Amino acid transport and metabolism
F	79	1.70	Nucleotide transport and metabolism
H	175	3.77	Coenzyme transport and metabolism
I	112	2.41	Lipid transport and metabolism
P	305	6.57	Inorganic ion transport and metabolism
Q	99	2.13	Secondary metabolites biosynthesis, transport and catabolism
R	563	12.12	General function prediction only
S	363	7.82	Function unknown
-	934	20.11	Not in COGs

The total is based on the total number of protein coding genes in the annotated genome.

Conclusion

On the basis of phenotypic, phylogenetic and genomic analyses, we formally propose the creation of *Enterobacter massiliensis* sp. nov. that contains strain JC163^T. This bacterium was cultivated from a healthy Senegalese individual, from whom several other previously undescribed bacterial species were also cultivated through diversification of culture conditions [4-15], thus suggesting that the human fecal flora from humans remains partially unknown.

Description of *Enterobacter massiliensis* sp. nov.

Enterobacter massiliensis (mas.il.i.en'sis. L. gen. masc. n. *massiliensis*, of Massilia, the Latin name of Marseille where strain JC163^T was first isolated and cultivated).

Colonies are 2 mm in diameter on Brain-Heart Infusion agar and are convex, opaque, light-cream colored and circular with regular margins. Cells are rods with tufts of polar flagella and a mean diameter of 1.02 µm and a mean length of 1.90 µm. Optimal growth is achieved in an aerobic atmosphere supplemented with 5% CO_2. Weak growth is observed in microaerophilic conditions. No growth is observed under anaerobic conditions in the absence of CO_2. Growth occurs between 25 and 45°C, with optimal growth occurring between 30 and 37°C. Cells stain Gram-negative, are non-endospore forming and are motile. Cells are positive for catalase and indole production. β-galactosidase and glucose, mannitol, sorbitol and rhamnose fermentation activities are present. Nitrate reduction, urease and oxidase activities are absent. Cells are susceptible to ticarcillin, imipenem, trimethoprim/sulfamethoxazole, gentamicin, amikacin, and colimycin, but resistant to fosfomycin and nitrofurantoin. The G+C content of the genome is 55.1%. The 16S rRNA and genome sequences are deposited in Genbank and EMBL under accession numbers JN657217 and CAEO00000000, respectively. The type strain JC163^T (= CSUR P161 = DSM 26120) was isolated from the fecal flora of a healthy patient in Senegal.

Acknowledgements

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