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Clostridium pacaense*: a new species within the genus *Clostridium

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Running title: *Clostridium pacaense*

Keywords: Culturomics, taxono-genomics, *Clostridium pacaense*

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5

6 **Abstract**

7 Using the taxonogenomic strategy, we described *Clostridium pacaense* sp. nov. strain
8 Marseille-P3100^T, a Gram-variable, non-motile, spore-forming anaerobic bacillus. This strain
9 was isolated from a 3.3-months-old Senegalese girl, with clinical aspects of marasmus. The
10 closest species based on 16S rRNA was *Clostridium aldenense* with a similarity of 98.4%.
11 The genome length was 2,672,129 bp, with a 50% of GC content and 2,360 proteins were
12 predicted. Finally, predominant fatty acids were Hexadecanoic acid, Tetradecanoic acid, 9-
13 Hexadecenoic acid.

14 **1. Introduction**

15 Human intestinal flora is incorporated mainly in the terminal part of small intestine and
16 colon. It consists of about 100,000 billion bacteria, grouped into 500 species including 90%
17 of anaerobic bacteria [1,2]. Oxygen-tolerant species such as lactobacilli, thus aerobic
18 organisms such as *E. coli* and enterococci represent minorities within intestinal microbiota
19 [3]. It appears that each adult own a signature of microbial community, and increasingly
20 influenced on human health [4–6]. Clostridiaceae is a family of Clostridia and has
21 traditionally been described by anaerobic growth and spore formation [4,7]. Clostridia
22 included major composition of mammalian gastrointestinal tract microbiomes [8]. For that
23 purpose, culturomics combined with taxonogenomics revealed main tools for the isolation
24 and characterization of new bacterial species. These techniques allowed the accessibility to
25 study their phenotypes like antibiotics resistance and biochemical features, thus genomics
26 characteristics and the potential impact on human health [9,10]. In the present work, we
27 propose *Clostridium pacaense* sp. nov. strain Marseille-P3100^T (CSUR P3100) as a new
28 species within the *Clostridium* genus. This strain was isolated from a 3.3-months-old
29 Senegalese girl, with clinical aspects of marasmus [11].

30

31 **2. Materials and Methods**

32 **2.1. Phenotypic, biochemical and antibiotics susceptibility**

33 Gram staining, motility, catalase and oxidase were determined as described by Lagier et al.
34 [12]. Sporulation was tested using a thermal shock on bacterial colonies (diluted in phosphate
35 buffered saline) for 20 minutes at 80°C. For electronic microscopy, a colony was collected
36 from agar and immersed into a 2.5 % glutaraldehyde fixative solution. The slide was gently
37 washed in water; air dried and examined with approximately 60 centimeters in height and 33
38 cm in width to evaluate bacteria structure on TM4000 microscope. Mass spectrum was
39 obtained from *C. pacaeense* colonies using MALDI-TOF (Figure 1). Biochemical
40 characteristics were tested using API 50CH, API ZYM and API 20A strips (bioMérieux,
41 Marcy l'Etoile, France). Antibiotic susceptibility referred to European Committee on
42 Antimicrobial Susceptibility Testing 2018 recommendations.

43 **2.2. Fatty acid methyl ester analysis**

44 Cellular fatty acid methyl ester (FAME) analysis was performed by GC/MS. Two samples
45 were prepared with approximately 35 mg of bacterial biomass per tube harvested from
46 several culture plates. Fatty acid methyl esters were prepared as described previously [13].
47 GC/MS analyses were carried out as described before [14]. Briefly, fatty acid methyl esters
48 were separated using an Elite 5-MS column and monitored by mass spectrometry (Clarus 500
49 - SQ 8 S, Perkin Elmer, Courtaboeuf, France). Spectral database search was performed using
50 MS Search 2.0 operated with the Standard Reference Database 1A (NIST, Gaithersburg,
51 USA) and the FAMEs mass spectral database (Wiley, Chichester, UK).

52 **2.3. Genome sequencing, assembly and annotation**

53 Genomic DNA was sequenced on MiSeq sequencer (Illumina, Inc., San Diego, CA) using
54 paired-end strategy as described previously [7]. Spades software was used for genome
55 assembly [15]. Contaminations were eliminated after performing BLASTn. Open reading

56 frames (ORFs) were predicted and annotated using Prokka software [16]. *C. pacaense*
57 genome was for protein functions against the Clusters of Orthologous Groups (COG)
58 database using BLASTP (E-value of $1e^{-03}$, coverage 0,7 and identity percent 30%). The
59 genome is available on EMBL-EBI scaffolds accession numbers: LS999944-LS999965.

60 **2.4. Comparative genomics**

61 Species to be compared were those with higher similarity based on 16s RNA (Figure 2),
62 provided the genome is available. The following bacterial species were used in this analysis
63 and their genomics features are summarized in supplementary Table S1: *Clostridium bolteae*
64 (GCA_002234575.2), *Clostridium lavalense* (GCA_003024655.1), *Clostridium*
65 *saccharolyticum* (GCA_000144625.1), *Clostridium aldenense* (GCA_003434055.1),
66 *Lachnospirillum citroniae* (GCA_000233455.1), *Clostridium amygdalinum*
67 (GCA_900205965.1), *Clostridium celerecrescens* (GCA_000732605.1). Amino-acids and
68 ORF sequences were predicted using Prodigal software to obtain optimized prediction within
69 all genomes [17]. Then, for each couple of genomes, a similarity percentage was computed
70 using the OrthoANI software [18].

71 **3. Results**

72 **3.1. Phenotypic and biochemical characterization**

73 *C. pacaense* is a Gram-variable, spore-forming, non-motile, anaerobic bacillus, with no
74 catalase and oxidase activities. Electron microscopy revealed that its length was 3.5 μm and
75 0.5 μm of diameter (Figure 3). *C. pacaense* produced α -glucosidase and Naphthol-AS-BI-
76 phosphohydrolase. General feature and biochemical characteristics are summarized in Table
77 1. Antibiotic susceptibility testing revealed that *C. pacaense* was susceptible to amoxicillin,
78 amoxicillin–clavulanic acid, ceftriaxone, ceftazidime, cefepime, ertapenem, metronidazole
79 and vancomycin.

80 **3.2. Predominant fatty acids**

81 The major fatty acids were Hexadecanoic acid (59 %), Tetradecanoic acid (20 %) and 9-
82 Hexadecenoic acid (9 %). No branched structures were detected (Table 2).

83 **3.3. Genomes properties and comparison**

84 *C. pacaense* draft genome consisted of 22 scaffolds, genome length was 2,672,129 bp, with a
85 50% of GC content. 2,360 proteins were predicted. The draft genome sequence of *C.*
86 *pacaense* owned the smallest genome. Its GC content was same as *C. aldenense*, but smaller
87 than *C. lavalense* and greater than others. Additionally, *C. pacaense* owned the smallest
88 number of predicted genes. Carbohydrate transport and metabolism thus secondary
89 metabolites biosynthesis, transport and catabolism were the predominant COG categories
90 identified within *C. pacaense* (Table 3). Based on 16s RNA similarity the closest species was
91 *C. aldenese* (Table 4). This was in agreement with genome data as *C. aldenese* was also the
92 closest species with OrthoANI value of 89.9744 % (*C. aldenese*) but below the 95% cut-off
93 defining a species (Figure 4).

94 **Description of *Clostridium pacaense* sp. nov.**

95 *Clostridium pacaense* (pa.ca.en'se L. masc. adj. *pacaense*, of PACA, the abbreviation of
96 Provence Alpes Cote d'Azur, the French area where the strain was isolated). In addition to
97 the characteristics in the genus description, cells are Gram-variable with a length of 3.5 µm
98 and a width of 0.5 µm. Produces α-glucosidase and Naphthol-AS-BI-phosphohydrolase. The
99 major fatty acids are C₁₆H₃₂O₂, C₁₄H₂₈O₂ and C₁₆H₃₀O₂. The type strain Marseille-P3100^T
100 has been deposited in the CSUR and CCUG culture collections under numbers CSUR P3100
101 and CCUG 71489, respectively. The type strain was isolated from stool sample of a
102 Senegalese girl with marasmus. The draft genome of the type strain is 2,672,129 bp long

103 with a DNA G + C content of 50% and is available on EMBL-EBI scaffolds accession
104 numbers: LS999944-LS999965.

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113 **Conflict of interest**

114 The authors have no conflict of interest to declare.

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176 **Table 1: General feature and biochemical tests of *Lachnoclostridium pacaense***

<p>Current classification</p>	<p>Domain: Bacteria Phylum: Firmicutes Class: Clostridia Order: Clostridiales Family: Clostridiaceae Genus: <i>Clostridium</i> Species: <i>Clostridium pacaense</i> Type strain: Marseille-P3100^T</p>
<p>Gram staining:</p> <p>Cell shape:</p> <p>Diameter:</p> <p>Cell length:</p> <p>Motility:</p> <p>Sporulation:</p> <p>Indole:</p>	<p>Variable</p> <p>Bacillus</p> <p>0.5 µm</p> <p>3.5 µm</p> <p>No</p> <p>Yes</p> <p>No</p>

Production of:

Alkaline phosphatase:	No
Catalase:	No
Oxidase:	No
Nitrate reductase:	No
Urease:	No
β -Galactosidase:	No
α -glucosidase	Yes
N-acetyl-glucosamine:	No
Esterase:	No

Acid from:

L-Arabinose:	<u>No</u>
Ribose:	<u>No</u>
Mannose:	<u>No</u>
Mannitol:	No
Sucrose:	No

D-Glucose:	No
D-Fructose:	No
D-Maltose:	No
D-Lactose:	No

177

178 **Table 2: Cellular fatty acids of *Clostridium pacaense***

Fatty acids	Name	Mean relative % ^a
16:0	Hexadecanoic acid	58.5 ± 0.5
14:0	Tetradecanoic acid	19.7 ± 0.3
16:1n7	9-Hexadecenoic acid	8.9 ± 0.2
18:1n9	9-Octadecenoic acid	5.5 ± 0.2
18:1n7	11-Octadecenoic acid	4.4 ± 0.3
18:0	Octadecanoic acid	1.0 ± 0.1
15:0	Pentadecanoic acid	TR
16:1n9	7-Hexadecenoic acid	TR

12:0

Dodecanoic acid

TR

179 ^aMean peak area percentage; TR = trace amounts < 1 %

180

181 **Table 3: *Clostridium pacaense* number of genes associated to COGs categories**

COGs description	Total
C: Chromatin structure and dynamics	119
D: Cell cycle control, mitosis and meiosis	17
E: Amino acid transport and metabolism	110
F: Nucleotide transport and metabolism	48
G: Carbohydrate transport and metabolism	280
H: Coenzyme transport and metabolism	44
I: Lipid transport and metabolism	31
J: Translation	41
K: Transcription	169
L: Replication, recombination and repair	73
M: Cell wall/membrane biogenesis	73
N: Cell motility	18
O: Posttranslational modification, protein turnover, chaperones	28

P: Inorganic ion transport and metabolism	76
Q: Secondary metabolites biosynthesis, transport and catabolism	7
R: General function prediction only	222
S: Function unknown	98
T: Signal transduction mechanisms	93
U: Intracellular trafficking and secretion	4
V: Defense mechanisms	55

182

183

184 **Table 4: *Clostridium pacaense* matrix of similarity based on 16S rRNA gene**

	<i>C. pacaense</i>	<i>C. lavalense</i>	<i>C. citroniae</i>	<i>C. celerecrescens</i>	<i>C. bolteae</i>	<i>C. amygdalinum</i>	<i>C. aldenense</i>	<i>C. saccharolyticum</i>
<i>C. pacaense</i>	ID							
<i>C. lavalense</i>	96.3	ID						
<i>C. citroniae</i>	96.7	96.1	ID					
<i>C. celerecrescens</i>	93.7	92.9	93.5	ID				
<i>C. bolteae</i>	95.7	97	96.8	94.1	ID			
<i>C. amygdalinum</i>	94.2	93.2	93.7	97.9	94.3	ID		
<i>C. aldenense</i>	98.4	95.9	96.7	93.9	95.8	94.1	ID	
<i>C. saccharolyticum</i>	94.2	93.2	93.6	98.5	94.1	98.8	94	ID

185 **16S rRNA sequences were aligned and similarity matrix were calculated using Bioedit Software [19].**

186 **Figure 1: Reference mass spectrum (MALDI-TOF) from *Clostridium pacaense* strain Marseille-P3100**

187 **Figure 2: Phylogenetic tree analysis based on 16S rRNA gene sequences**

188 16S rRNA genes were aligned using CLUSTALW and phylogenetic tree was generated using MEGA 7 software [20]

189 **Figure 3: Electron microscopy of *Clostridium pacaense***

190 **Figure 4: OrthoAni Heatmap of implicated genomes**







