Cloacibacillus evryensis gen. nov., sp. nov., a novel asaccharolytic, mesophilic, amino-acid-degrading bacterium within the phylum 'Synergistetes', isolated from an anaerobic sludge digester

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A novel anaerobic, mesophilic, amino-acid-utilizing bacterium, strain 158^T, was isolated from an anaerobic digester of a wastewater treatment plant. Cells of strain 158^T were non-motile, rod-shaped (2.0–3.0 × 0.8–1.0 µm) and stained Gram-negative. Optimal growth occurred at 37 °C and pH 7.0 in an anaerobic basal medium containing 1 % Casamino acids. Strain 158^T fermented arginine, histidine, lysine and serine and showed growth on yeast extract, brain-heart infusion (BHI) medium and tryptone, but not on carbohydrates, organic acids or alcohols. The end products of degradation were: acetate, butyrate, H₂ and CO₂ from arginine; acetate, propionate, butyrate, H₂ and CO₂ from lysine; and acetate, propionate, butyrate, valerate, H₂ and CO₂ from histidine, serine, BHI medium, Casamino acids and tryptone. The DNA G+C content was 55.8 mol%. The 16S rRNA gene sequence of strain 158^T showed only 92.6 % sequence similarity with that of *Synergistes jonesii*, the only described species of the 'Synergistes' group. The major cellular fatty acids were iso-C_{15:0} (16.63 %), iso-C_{15:0} 3-OH (12.41 %) and C_{17:1ω6c} (9.46 %) and the polar fatty acids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylmonomethylamine; these fatty acid profiles did not resemble those of any recognized bacterial species. Due to the considerable differences in genotypic, phenotypic and phylogenetic characteristics between strain 158^T and those of its nearest relative, it is proposed that strain 158^T represents a novel species in a new genus, *Cloacibacillus evryensis* gen. nov., sp. nov., in the phylum 'Synergistetes'. The type strain is 158^T (=DSM 19522^T=JCM 14828^T).

Proteins, as well as carbohydrates and lipids, comprise a major part of the organic load in anaerobic sludges and wastewaters. For example, the protein component of dairy wastewater and domestic sewage is reportedly greater than

40 % (Ramsay & Pullammanappallil, 2001). Protein hydrolysis in anaerobic environments has not been well studied except in the animal gut, e.g. the rumen (Debroas & Blanchart, 1993), and has been restricted to molecular inventories in protein-containing wastewaters (Tang *et al.*, 2005). The major difference between protein degradation in the rumen and in anaerobic digesters is that, in the rumen, the ruminal bacteria (both saccharolytic and asaccharolytic) grow on amino acids and peptides as major

Abbreviations: CFA, cellular fatty acid; DHP, 3,4-dihydroxypyridine; SGOs, *Synergistes* group of organisms; VFA, volatile fatty acid; WWTP, wastewater treatment plant.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Cloacibacillus evryensis* strain 158^T is CU463952.

energy substrates (Eschenlauer *et al.*, 2002). In anaerobic reactors, however, proteolytic bacteria predominantly mediate protein degradation and the process involved is energy yielding (Ramsay & Pullammanappallil, 2001).

Under methanogenic conditions, proteins are hydrolysed to peptides and amino acids, which are subsequently fermented to volatile fatty acids (VFAs) and finally to methane and CO₂ by mixed microbial populations. Species from genera such as *Clostridium* (Paster *et al.*, 1993), *Peptostreptococcus* (Cato *et al.*, 1983), *Acidaminobacter* (Stams & Hansen, 1984), *Aminomonas* (Baena *et al.*, 1999), *Aminobacterium* (Baena *et al.*, 1998, 2000), *Thermanaerovibrio* (Zavarzina *et al.*, 2000; Guangsheng *et al.*, 1992), *Eubacterium* (Zindel *et al.*, 1988), *Dethiosulfovibrio* (Magot *et al.*, 1997), *Aminiphilus* (Díaz *et al.*, 2007), *Thermovirga* (Dahle & Birkeland, 2006), *Jonquetella* (Jumas Bilak *et al.*, 2007) and *Synergistes* (Allison *et al.*, 1992) have been shown to degrade amino acids anaerobically.

In the last edition of *Bergey's Manual of Systematic Bacteriology* (Garrrity & Holt, 2001), the *Synergistes* group under the class *Deferribacteres* was represented by a number of *genera incertae sedis*, whose taxonomic positions have not been well-defined, with only one described phylotype, namely *Synergistes jonesii*. Using 16S rRNA gene targeted PCR assays, Godon *et al.* (2005) explored 93 anaerobic environments and reported that members of the *Synergistes* group of organisms (SGOs) were present in 95 % of the ecosystems analysed and the sequences formed separate phylogenetic groups, although their proportion was generally below 1 %. Also, Horz *et al.* (2006) have found that SGOs are ubiquitous in humans and have been detected in various infectious diseases. They identified clinically relevant isolates that make up three unique clusters that apparently occupy their own individual ecological niches within the human body such as peritoneal fluid, soft tissues and periodontal pockets, the latter having no isolated representatives. Although several isolates from human clinical samples have been added to the list recently (Vartoukian *et al.*, 2007; Jumas-Bilak *et al.*, 2007), to date, *S. jonesii* is the only isolate among the SGOs that has been studied and fully characterized (Allison *et al.*, 1992). Also, there is no isolated representative of the SGOs from anaerobic digesters; thus, the origin and role of these organisms in wastewater treatment plants (WWTPs) remain unknown. In this paper, the isolation and description of strain 158^T, which emerges as a novel member in the phylogenetic line represented by the genus *Synergistes*, from a mesophilic anaerobic digester of a WWTP are reported.

The bacterial strain described in this study was isolated from a mesophilic anaerobic digester of a WWTP treating municipal wastewater in Evry (250 000 population equivalents), 35 km south of Paris, France. The digester working parameters were: temperature, 33 °C; pH, 7.2; and retention time, 37.5 days.

The Hungate technique (Hungate, 1969) was used for strain isolation. The basal medium used in the study contained (l⁻¹) 3 g KH₂PO₄, 3 g K₂HPO₄, 6 g NaCl, 6 g (NH₄)₂SO₄, 0.6 g CaCl₂, 1.23 g MgSO₄·7H₂O and 1 g sodium citrate supplemented with 2 g yeast extract, 10 g Casamino acids and 10 ml trace element solution (Balch *et al.*, 1979). The pH was adjusted to 7.0 with 10 M KOH. The medium was boiled under O₂-free N₂ gas, cooled, sparged with N₂/CO₂ (80:20, v/v), autoclaved at 121 °C for 20 min and reduced with sterile sulfide/cysteine solution prior to inoculation. Enrichments were incubated at 33 °C and examined for growth with a phase-contrast microscope. Enrichment cultures were transferred periodically into fresh medium with 10 % (v/v) inocula. Bacterial growth was evident on Casamino acids-based medium at 33 °C after 5–7 days of growth under anaerobic conditions. Microscopic examination of this primary enrichment culture revealed micro-organisms with diverse morphologies (data not shown), but after several sub-cultures, small rod-shaped cells were dominant. Isolation was performed in plates with the same medium solidified with agar (2 %) and incubated in anaerobic jars at 33 °C with N₂/CO₂ (80:20, v/v) as gas phase at 2 atm (203 kPa) pressure. After 10 days incubation, small (about 2 mm in diameter), light brown, transparent, lens-shaped colonies with smooth margins appeared predominantly on the solid agar. Isolates were purified by repeated isolations until deemed to be axenic. One of the purified strains, designated strain 158^T, was taken up for further characterization. Subsequent routine culturing of strain 158^T was performed using basal medium containing 1 % (w/v) Casamino acids and 0.2 % (w/v) yeast extract.

Light microscopy was performed using a Zeiss axioplan phase-contrast microscope. Cells of strain 158^T grown in Casamino acid medium were non-motile rods, occurring singly or in pairs (Fig. 1a). Cells were variable in size, with longer cells (2.0–3.0 × 0.8–1.0 µm) being observed in older cultures. Long chains were observed in 15-day-old cultures. Motility and spores were never observed and the culture did not survive heat treatment for 2 h at 80 °C. For electron microscopic studies, the culture was centrifuged at 3000 g for 6 min at 4 °C, the supernatant was discarded and the pellet was fixed in 3 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature. Samples were then post-fixed in 1 % osmium tetroxide for 1 h and washed again in buffer prior to being dehydrated in an ascending gradient of ethanol [50, 70, 80, 90, 95 and 100 % (v/v)]. Finally, they were embedded in Epon resin. Ultrathin sections were stained with 2 % (w/v) uranyl acetate and examined with a Tecnai F20 transmission electron microscope (Philips) operating at 200 kV. Cells of strain 158^T stained Gram-negative and ultrathin sections revealed a typical multilayered Gram-negative cell wall structure (Fig. 1b).

Growth experiments were conducted in duplicate in basal medium containing 1 % (w/v) Casamino acids and 0.2 % (w/v) yeast extract. All media were inoculated [10 % (v/v)]

Fig. 1. (a) Phase-contrast photomicrography of cells from the exponential growth phase of strain 158^T; bar, 5 µm. (b) Transmission electron micrograph of ultrathin section of cells of strain 158^T showing a typical multilayered Gram-negative cell-wall structure (OW, outer cell wall; PG, peptidoglycan layer; CM, cytoplasmic membrane); bar, 0.2 µm.

with late phase exponential cultures grown on Casamino acids and yeast extract. Strain 158^T was a strict anaerobe, as shown by its inability to grow on Casamino acid agar under aerobic conditions. Under anaerobic conditions, the addition of 0.2% yeast extract enhanced growth, although growth occurred even in the absence of yeast extract. For pH studies, the medium was adjusted with anaerobically prepared stock solutions of 1 M HCl or 10% (w/v) NaOH to initial pH values of 4.0–10.0. The pH range for growth at 37 °C was 6.5–10.0, with optimum growth at pH 7.0–7.5. Growth at different temperatures was determined at 15–55 °C. The temperature range for growth at pH 7.0 was 20–50 °C, with optimal growth at 37–40 °C. The doubling time under optimal conditions on Casamino acid medium was 15 h. NaCl requirements for growth were tested at concentrations between 0.1 and 2.0% (w/v) by weighing NaCl directly into vials before dispensing the medium. Strain 158^T tolerated up to 0.7% NaCl for growth, but growth was inhibited at higher concentrations.

Substrate utilization was determined in triplicate on pre-reduced basal medium with 0.2% (w/v) yeast extract and N₂ as the gas phase. Soluble substrates were added from

sterilized anaerobically prepared stock solutions (1 M), whereas insoluble substrates were added directly to the vial before the media was dispensed. Growth was measured at 600 nm in an Ultraspec 2000 (Pharmacia Biotech) spectrophotometer. Head-space gas analysis was performed using a micro GC (CP4900; Varian) equipped with four parallel chromatographic lines (two molecular sieve 5A, a poraplot Q and volamine columns) coupled with a thermo-conductivity detector. The poraplot Q column was used for CO₂, N₂O and H₂S (helium as carrier gas, column temperature of 30 °C, injector temperature of 50 °C), the first molecular sieve column was for N₂ and CH₄ (helium as carrier gas, column temperature of 55 °C, injector temperature of 50 °C), the second molecular sieve was for H₂ (argon as carrier gas, column temperature of 55 °C, injector temperature of 50 °C) and the volamine was for NH₃ (helium as carrier gas, column temperature of 55 °C, injector temperature of 50 °C). The standard calibration was made with gas mixtures containing (v/v) 0.5% H₂S, 3% N₂O, 40% CO₂, 50% CH₄, 5% H₂ and 6.5% N₂ (Air Products). VFAs – acetic, butyric, propionic and valeric acids – were analysed by GC (Thermoquest TRACE GC2000) equipped with a flame-ionization detector and a DB-WAXetr column (length 30 m, inner diameter 0.53 mm, film thickness 1 µm). Injector and detector temperatures were 230 °C and 300 °C, respectively. Helium (4 ml min⁻¹) was the carrier gas. Growth and product formation were analysed after 2–3 weeks of incubation at 37 °C. Table 1 shows the substrates used by strain 158^T and the end products formed. Growth was enhanced by yeast extract and strain 158^T fermented histidine, arginine, lysine, serine and tryptophan (10 mM each). Poor growth was observed on alanine, glutamate, aspartate, proline, glycine, cysteine and phenylalanine, whereas no growth was observed on isoleucine, leucine, valine, threonine, methionine, glutamine or asparagine. Strain 158^T was also able to grow on complex substrates (0.5%, w/v) such as brain-heart infusion (BHI), tryptone and yeast extract, but not on phytone-peptone or tryptic soy broth. None of the sugars, organic acids or alcohols (10 mM each) tested were utilized apart from mannose, rhamnose and α-ketoglutarate, although only faint growth was observed on these. Also, strain 158^T did not grow on non-protein amino acids such as mimosine and 3,4-dihydroxypyridine (DHP) at a final concentration of 10 mM.

The trait of degrading proteinaceous compounds alone observed in strain 158^T is a property shared with only a few members of the 'Firmicutes', such as *Clostridium pascui*, *C. acetireducens*, *C. litorale*, *C. sticklandii*, *C. aminophilum*, *Sedimentibacter hydroxybenzoicum* (formerly *Clostridium hydroxybenzoicum*), *Peptostreptococcus anaerobius*, *Acidaminobacter hydrogenoformans*, *Eubacterium acidaminophilum*, *Anaeromusa acidaminophila* (Baena *et al.*, 1999), and those in the phylum 'Synergistetes', namely *Aminomonas paucivorans*, *Aminobacterium colombiense*, *Aminobacterium mobile*, *Dethiosulfovibrio peptidovorans*,

Table 1. Fermentation of substrates that support growth of strain 158^T in pure culture

Poor growth was observed on alanine, glutamate, aspartate, proline, glycine, cysteine and phenylalanine and acetate levels above those of control were present. Tubes containing basal medium, 0.2 % (w/v) yeast extract and inoculum, but lacking substrates were used as controls.

Substrate	Products*						Maximum OD ₆₀₀
	Acetic acid	Propionic acid	Butyric acid	Valeric acid	CO ₂ †	H ₂ †	
Casamino acids (1 %)	36.3	2.01	2.76	0.53	22	3.5	0.768
BHI (0.5 %)	23.26	3.53	9.44	1.15	3.4	1.4	0.76
Tryptone (0.5 %)	17.78	2.2	5.71	0.74	4.5	4.5	0.133
Arginine (10 mM)	27.53	0	55.6	0	5.9	4.1	0.162
Histidine (10 mM)	44.06	2.74	2.09	3.53	3.8	3.0	0.317
Arginine + histidine (10 + 20 mM)	4.9	0.76	4.95	0	6.4	4.7	0.105
Lysine (10 mM)	18.02	0.46	1.46	0	2.2	4.9	0.10
Serine (10 mM)	19.43	0.49	3.6	0.75	3.3	4.8	0.103
Tryptophan (10 mM)	22.43	0	0	0	1.8	1.3	0.092

*All values were corrected for the small amount of acetate formed in the control tubes. Experiments were conducted in triplicate and mean values (in mM, except where indicated) are given; standard deviations were always within 5 % of the mean value.

†Values represent percentages (v/v) in the gas phase. Trace amounts of ammonia were also detected for some substrates; the remaining gas is N₂ already present in the gas phase.

Jonquetella anthropi and *S. jonesii*, etc. (Vartoukian *et al.*, 2007). Very little is understood about biochemical pathways operating in obligately anaerobic aminolytic bacteria. Strain 158^T fermented arginine to acetate and butyrate, whereas *S. jonesii* produced ornithine and citrulline in addition to acetate and butyrate (McSweeney *et al.*, 1993); *Thermanaerovibrio acidaminovorans* fermented arginine to acetate and propionate only in co-culture with *Methanobacterium thermoautotrophicum* (Guangsheng *et al.*, 1992), whereas acetate, formate and ornithine were reported for *Aminomonas paucivorans* (Baena *et al.*, 1999). Strain 158^T degraded histidine to acetate, propionate, butyrate and valerate. This is different from the situations observed in *S. jonesii* (McSweeney *et al.*, 1993), which ferments histidine to propionate, acetate, ornithine and butyrate, and *Aminomonas paucivorans*, which ferments histidine to acetate and formate (Baena *et al.*, 1999). However, the presence of valerate as one of the fermentation products of histidine has not been reported up till now, although this could have close similarities with the scheme proposed for fatty acid elongation in the rumen bacterium *Eubacterium pyruvativorans* (Wallace *et al.*, 2004). It is worthwhile to mention here that isovalerate was observed as one of the end products of fermentation of trypticase-glucose-yeast extract broth in *J. anthropi*, another member of the phylum 'Synergistetes' (Jumas-Bilak *et al.*, 2007). The conversion of lysine to acetate and butyrate (Table 1) by strain 158^T suggests a pattern similar to that reported for anaerobic asaccharolytic Gram-positive rods such as *Clostridium peptidovorans* (Mechichi *et al.*, 2000) and species of the genus *Eubacterium* that inhabit periodontal pockets (Uematsu *et al.*, 2003). These micro-organisms produce ATP by degrading lysine to acetate as

observed from their acetate kinase activities (Uematsu *et al.*, 2003). Minor amounts of propionate were also detected during the fermentation of lysine by strain 158^T and, hence, further investigation into lysine degradation by this strain is required. Serine was fermented to acetate and butyrate (Table 1), with minor amounts of propionate and valerate, similar to that reported for a Gram-negative coccus (strain LC3) isolated from the rumen of sheep (Lewis & Elsdén, 1955). This again is of considerable interest as strain 158^T and LC3 produce VFAs longer than butyrate. Clearly, the metabolism of serine by strain 158^T requires further investigation. Growth on tryptophan was only moderate and acetate appeared to be the only fermentation product.

The effect of different gas phases on growth of the isolate was tested on the substrates that supported growth of strain 158^T. An 80 % hydrogen atmosphere (supplied as 80:20 H₂/CO₂) at 2 atm (203 kPa) pressure inhibited growth of strain 158^T on Casamino acid medium to a minor extent, whereas growth in nitrogen or N₂/CO₂ was not inhibited. Growth was not inhibited on amino acids under a hydrogen atmosphere. The electron acceptors nitrate, sulfate, thiosulfate (all at 10 mM), sulfite (2 mM) and fumarate (20 mM) were tested on all substrates. The addition of various electron acceptors did not increase the range of amino acids utilized and did not significantly improve growth on alanine, arginine, histidine, lysine or serine with respect to controls. Amino acid degradation via the Stickland reaction was determined in the basal medium containing the following amino acid couplets as electron donors and acceptors, respectively (10 mM/20 mM): alanine/arginine, alanine/proline, alanine/serine, alanine/glycine, alanine/tryptophan, leucine/glycine, isoleucine/

glycine, valine/glycine and arginine/histidine. Strain 158^T did not perform the Stickland reaction when grown on any of the amino acid couplets tested. Even though growth was observed on arginine/histidine, it was less than that on each of the individual amino acids (Table 1).

Antibiotic resistance was determined in mineral base medium with 0.2% (w/v) yeast extract at pH 7.0 and 37 °C. Antibiotics were filter-sterilized and the following concentrations were tested per 50 ml medium: penicillin (10 mg), ampicillin (10 mg), kanamycin (10 mg) and vancomycin (5 µg). Growth of strain 158^T was not inhibited by vancomycin, although it was inhibited in the presence of penicillin, ampicillin and kanamycin. The DNA G + C content of strain 158^T was determined by the DSMZ, Braunschweig, Germany, as described by Mesbah *et al.* (1989). The method involves enzymic degradation of DNA and separation of the nucleosides by reverse-phase HPLC. The DNA G + C content of strain 158^T was 55.8 mol%.

The cellular fatty acid (CFA) and polar lipid composition were determined by the Identification Service of the DSMZ and B. J. Tindall, DSMZ, Braunschweig, Germany, by GC and two-dimensional TLC methods, respectively. The major CFAs (Table 2) of strain 158^T were iso-C_{15:0} (16.63%), iso-C_{15:0} 3-OH (12.41%) and C_{17:1}ω6c (9.46%); its closest relative, *S. jonesii*, showed different CFA ratios and lacked certain CFAs. CFAs in strain 158^T were compared by the Sherlock Microbial Identification (MIDI) system to the existing database on the basis of its unique fatty acid profile, but no matches to any known bacterial CFA profiles were found. This supports the affiliation of strain 158^T to a new genus. The polar fatty acid profile of strain 158^T (data not shown) revealed diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylmonomethylamine.

Genomic DNA was extracted from the liquid enrichment of the isolate as described by Chouari *et al.* (2003). The universal bacterial primers 008F and 1390R were used to obtain a PCR product of approximately 1.4 kb. DNA amplification, PCR thermal profiling and sequencing were performed as described by Chouari *et al.* (2003). The 16S rRNA gene sequence (1308 bp) of strain 158^T corresponded to positions 38–1384 of the *Escherichia coli* 16S rRNA gene and was compared with sequences from the complete EMBL nucleotide database. Sequences of nearest neighbours of cultured species from the EMBL database with the best BLAST scores were imported into the ARB database set (www.arb-home.de) and aligned automatically. The resulting alignments were checked manually and corrected where necessary. BLAST searches in international databases revealed that the most closely related sequences to that of strain 158^T were the 16S rRNA gene from a bacterium, ADV66, isolated from human vaginal flora (GenBank accession no. EF468684) and from *Synergistes* spp. isolated from human blood (EF551160, EF551161, EF551162) and peritoneal fluid samples (DQ412721, DQ412717, DQ412718) (Horz *et al.*, 2006) (Fig. 2).

Table 2. Cellular fatty acid profiles (%) of strain 158^T and *S. jonesii*

ω, Position of the double bond from the methyl end; c, *cis* isomer; cyc, cyclopropane; DMA, dimethyl acetal. Major fatty acids are given in bold type.

Fatty acid	Strain 158 ^T	<i>S. jonesii</i>
Saturated:		
C _{12:0}	0.2	—
C _{13:0}	2.22	2.0
iso-C _{13:0}	6.51	6.0
C _{14:0}	0.34	1.0
C _{14:0} DMA	0.63	—
C _{15:0}	2.63	9.0
iso-C _{15:0}	16.63	3.0
anteiso-C _{15:0}	0.36	1.0
C _{16:0}	2.05	3.0
C _{17:0}	6.7	14.0
iso-C _{17:0}	5.62	3.0
C _{17:0} DMA	0.83	—
C _{18:0}	1.22	3.0
Unsaturated:		
C _{15:1} ω6c	2.65	1.0
C _{16:1} ω7c	2.44	1.0
C _{17:1} ω6c	9.46	9.0
iso-C _{17:1}	—	1.0
C _{18:1} ω5c	5.5	1.0
C _{19:1} ω12c	0.65	7.0
iso-C _{19:1}	—	1.0
Hydroxy:		
C _{13:0} 3-OH	—	1.0
C _{14:0} 3-OH	—	1.0
iso-C _{15:0} 3-OH	12.41	7.0
C _{15:0} 3-OH	4.6	8.0
C _{16:0} 3-OH	0.49	—
Cyclopropane fatty acids:		
C ₁₉ cyc9,10	1.39	1.0
C ₁₉ cyc11,12	1.15	—
C ₂₀ cyc	—	14.0
Summed features: *		
C _{15:0} DMA/C _{14:0} 3-OH	2.03	—
anteiso-C _{15:0} 3-OH/C _{16:1} ω9c DMA	0.74	—
C _{17:2} at 16.760/C _{17:1} ω9c	6.81	—
C _{17:1} ω8c/C _{17:2} at 16.801	1.39	—
C _{18:1} ω7c/unknown at 17.834†	1.15	—
Unknown at 18.622†	1.16	—

*Summed features represent two fatty acids that cannot be separated by GLC with the MIDI system.

†Unknown fatty acids have no name listed in the peak library file of the MIDI system and therefore cannot be identified; values are equivalent chain-lengths.

Strain 158^T grouped with the uncultured clones of *Synergistes* group 8 (data not shown) from anaerobic digesters (Godon *et al.*, 2005), albeit with only 90% similarity to the most closely related sequence, clone MLE1-42 (AF280863).

Fig. 2. Evolutionary dendrogram constructed by the neighbour-joining method showing the affiliation of strain 158^T to its closest relatives in the GenBank database. The sequence from *Bacteroides intestinalis* was used as the outgroup to root the tree. Bootstrap values of 100 resamplings are shown for the closest relatives. Only bootstrap values above 70% were considered significant and reported. Bar, 10 nucleotide substitutions per 100 nucleotides.

Phylogenetic trees were constructed using the ARB program and database package. Placement was done by comparison with reference sequences representing the main lines of descent in the domain *Bacteria*. Phylogenetic trees based on the 16S rRNA gene sequences of cultured bacteria in the neighbourhood of strain 158^T were built using three methods provided by PAUP 4.0b10 software (Swofford, 2002): distance, maximum-likelihood and maximum-parsimony. A strict consensus tree was drawn when multiple best trees were obtained. The delineation of species, genus and family within groups of *Synergistes* was

proposed by using distance values of 0.03 to differentiate at the species level, 0.05 at the genus level and 0.1 at the family/class level (Schloss & Handelsman, 2004). In the phylogenetic trees obtained with the three building methods, the cultured *Synergistes* spp. formed two monophyletic groups supported by high bootstrap values, rooted by the 16S rRNA gene of *S. jonesii* (Fig. 2). Strain 158^T was clearly included in group 'OTU1' formed by isolates NML05A017, RMA14605, RMA16088 and ADV66 of human origin. Sequences within OTU1 shared a high mean similarity value of 98.83% (or a low distance value of

0.015) and were in agreement with the criteria generally admitted to limit the species, that is, 97 % similarity and a distance value of up to 0.03. Group OTU1 had a mean similarity of 95.15 % (distance value of 0.069) with sequences from group 'OTU2', represented by isolates NML060450, NML96A088 and RMA 16290, also of human origin. The level of similarity and distance between groups OTU1 and OTU2 was similar to that observed between *Aminobacterium mobile* and *Aminobacterium colombiense* (94.69 % for similarity; 0.074 for distance), two species of the same genus. *S. jonesii* was more distantly related to the sequences in OTU1 and OTU2 (92.36 and 92.7 % similarities, respectively).

Several phenotypic properties also support this conclusion (Table 3). Strain 158^T shares with *S. jonesii* the ability to ferment arginine and histidine, yeast extract, BHI and Casamino acids; both are Gram-negative, non-motile, non-spore-forming rods that do not utilize carbohydrates or organic acids as carbon sources and do not perform the Stickland reaction. Strain 158^T differs from *S. jonesii* in its pH range for growth, which in turn may reflect their habitats. Strain 158^T was isolated from an anaerobic digester and, hence, exhibits growth in the alkaline range (up to pH 10) as is observed with other species isolated from anaerobic digesters such as *Aminobacterium colombiense* (Baena *et al.*, 1998) and *Aminobacterium mobile* (Baena *et al.*, 2000). *S. jonesii*, like all other rumen bacteria has a growth range of pH 6.0–6.9 (Kamra, 2005). In addition to arginine and histidine, strain 158^T fermented other amino acids such as lysine and serine, whereas *S. jonesii* did not (Table 2). Growth on non-protein amino acids such as mimosine or its degradation component 3,4-DHP was reported as a distinctive trait of *S. jonesii* (Allison *et al.*, 1992); however, this property was not observed in strain 158^T.

The low 16S rRNA gene sequence similarity levels between strain 158^T and *S. jonesii*, *Aminobacterium colombiense* and *Aminobacterium mobile* (92.58, 86.31 and 86.79 %, respectively) and its distinctive phylogenetic position allow us to affirm strain 158^T as a representative of a novel species in a new genus. Furthermore, the DNA G+C content of strain 158^T (55.8 mol%), which is slightly lower than that of *S. jonesii* (58 mol%), and its unique CFA profile (Table 2) support its affiliation in a new genus. Based on 16S rRNA gene sequence analysis and its phylogenetic position, it is proposed that the species represented by strain 158^T be given genus status in a newly proposed family 'Synergistaceae', which encompasses groups OTU1 and OTU2 and *S. jonesii* (Fig. 2). According to the Bergey's system of classification (Garrity & Holt, 2001), placement of *S. jonesii* in the phylum 'Deferribacteres' was provisional. Isolation of different *Synergistes*-like micro-organisms (Horz *et al.*, 2006) and the retrieval of many other *Synergistes* rRNA gene sequences from different environments have allowed a thorough phylogenetic analysis of this group of micro-organisms. Our study (Fig. 2) corroborates other studies (Godon *et al.*, 2005; Horz *et*

al., 2006; Vartoukian *et al.*, 2007; Jumas-Bilak *et al.*, 2007) showing that the SGOs and related genera are reproducibly monophyletic and unaffiliated with the *Flexistipes*–*Deferribacter* group and, therefore, the creation of a new phylum 'Synergistetes', as proposed by Jumas-Bilak *et al.* (2007), is justified.

With the isolation of strain 158^T, we extend our knowledge of the biodiversity of asaccharolytic amino-acid-degraders from anaerobic sludge digesters. On the basis of the phenotypic, genotypic and phylogenetic evidence presented and based on the current taxonomic guidelines, it is proposed that strain 158^T represents a novel species in a new genus, *Cloacibacillus evryensis* gen. nov., sp. nov., in a new family 'Synergistaceae', phylum 'Synergistetes'.

Description of *Cloacibacillus* gen. nov.

Cloacibacillus (Clo.a'ci.ba.cil.lus. L. n. *cloaca* a sewer canal; L. masc. n. *bacillus* a small staff; N.L. masc. n. *Cloacibacillus* a small staff from a sewer canal).

Cells are strictly anaerobic rods, non-motile, with no flagella. Gram-negative type cell wall. Mesophilic and non-spore-forming. Neutrophilic and heterotrophic in nature. Asaccharolytic and proteolytic. Metabolism is fermentative, does not reduce any of the electron acceptors and does not perform the Stickland reaction. The major fatty acids are iso-C_{15:0}, iso-C_{15:0} 3-OH and C_{17:1}ω6c. Members of this genus can be differentiated from other genera in the phylum 'Synergistetes' by 16S rRNA gene sequencing, CFA profiles, DNA G+C content and metabolic end products. The genus is affiliated to the family 'Synergistaceae' in the phylum 'Synergistetes'. The type species is *Cloacibacillus evryensis*.

Description of *Cloacibacillus evryensis* sp. nov.

Cloacibacillus evryensis (ev.ry.en.sis. N.L. masc. adj. *evryensis* pertaining to Evry, the origin of the isolate).

In addition to the properties listed in the genus description, the following properties are observed. Rod-shaped bacterium, 2.0–3.0 × 0.8–1.0 µm in size, occurring singly or in pairs; older cells form chains. Electron microscopy reveals a cell-wall structure typical of Gram-negative bacteria. Colonies are light brown, small (about 1–2 mm in diameter), transparent and lens-shaped with smooth margins. Mesophilic, optimum growth temperature is 37 °C and the growth range is 20–50 °C. The pH range for growth is 6.5–10.0, with optimum at pH 7.0. Doubling time is 15 h on Casamino acid medium. Tolerates less than 0.7 % NaCl for growth. Growth is enhanced by yeast extract. Ferments histidine, arginine, lysine, serine, BHI, yeast extract and tryptone. No growth is observed on other amino acids or carbohydrates. Acetate, butyrate, propionate, valerate, H₂ and CO₂ are the usual end products of fermentation. Sensitive to penicillin, ampicillin and kanamycin, but resistant to vancomycin.

Table 3. Differential characteristics of the genus *Cloacibacillus* and related genera in the phylum 'Synergistetes'

Genera: 1, *Cloacibacillus* (represented by *Cloacibacillus evryensis* gen. nov., sp. nov. strain 158^T); 2, *Synergistes* [represented by *S. jonesii* (GenBank accession no. L08066)]; 3, *Aminobacterium* [*Aminobacterium colombiense* (AF069287) and *Aminobacterium mobile* (AF073521)]; 4, *Aminomonas* [*Aminomonas paucivorans* (AF072581)]; 5, *Anaerobaculum* [*Anaerobaculum thermoterranum* (U50711) and *Anaerobaculum mobile* (AJ243189)]; 6, *Thermanaerovibrio* [*T. acidaminovorans* (AF071414) and *T. velox* (AF161069)]; 7, *Dethiosulfovibrio* [*D. peptidovorans* (U52817), *D. acidaminovorans* (AY005466), *D. russensis* (AF234544) and *D. marinus* (AF234542)]. +, Positive; −, negative; +/−, some species are positive and some are negative; ND, not determined.

Characteristic	1	2	3	4	5	6	7
Habitat	Anaerobic digester, human clinical samples	Goat rumen	Anaerobic dairy wastewater treatment plant	Anaerobic dairy wastewater treatment plant	Anaerobic digester	Anaerobic digester, cyanobacterial mat	Saline oil wells, sulfur mats
Cell morphology	Straight rods	Rods	Rods	Curved rods	Rods	Vibrios	Vibrios
Optimum growth temperature (°C)	35–40	39	37	37	55–60	55–65	28–42
DNA G + C content (mol%)	55.8	58	44–46	43	44.0–51.5	54–57	50–56
Motility	−	−	+ / −	−	+ / −	+	+
Metabolism	Fermentative	Fermentative	Fermentative	Fermentative	Fermentative/respiratory	Fermentative/respiratory	Fermentative/respiratory
Fermentation	Proteolytic	Proteolytic	Proteolytic	Proteolytic	Proteolytic and saccharolytic	Proteolytic and saccharolytic	Proteolytic
Electron acceptors	−	ND	−	−	Crotonate, thiosulfate, cysteine, sulfur	Elemental sulfur	Sulfur and thiosulfate
Stickland reaction	−	−	−	ND	ND	ND	ND
Major cellular fatty acids	iso-C _{15:0} , iso-C _{15:0} 3-OH, C _{17:1} ω6c (lacks C ₂₀ cyc)	C _{17:0} , C ₂₀ cyc, C _{17:1} ω6c	ND	ND	ND	ND	iso-C _{15:0} , C _{18:0} *
Additional features	Some species of this genus detected in human clinical infections	Degrades 3,4-DHP	Ferments some amino acids in co-culture with <i>Methanobacterium formicum</i>	Ferments some amino acids in co-culture with <i>Methanobacterium formicum</i>	Crotonate used as electron acceptor	Thermophilic	Moderately halophilic

*Data from Jumas-Bilak *et al.* (2007).

Does not grow on non-protein amino acids such as mimosine and 3,4-DHP.

The type strain, strain 158^T (=DSM 19522^T=JCM 14828^T), was isolated from an anaerobic digester of a WWTP in Evry, France. The DNA G+C content of the type strain is 55.8 mol%.

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