Oscillibacter valericigenes gen. nov., sp. nov., a valerate-producing anaerobic bacterium isolated from the alimentary canal of a Japanese corbicula clam

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A mesophilic, strictly anaerobic bacterium, strain Sjm18-20^T, was isolated from the alimentary canal of a Japanese corbicula clam. Cells of strain Sjm18-20^T were Gram-negative, non-sporulating, straight to slightly curved rods, 2.5–6.0 μm long, and were motile with oscillatory movements by means of peritrichous flagella. Cells elongated to 30 μm after prolonged cultivation. Optimum growth was observed at 30 °C and pH 6.0–6.5. Growth occurred below 4.0 % (w/v) NaCl. Strain Sjm18-20^T produced acid from D-glucose and a few pentoses such as L-arabinose, D-ribose and D-xylose. *n*-Valeric acid was the major end product from glucose. The genomic DNA G+C content of strain Sjm18-20^T was 52.9 mol%. Phylogenetic analysis based on the 16S rRNA gene revealed that strain Sjm18-20^T could be accommodated in clostridial cluster IV of the low-G+C-content Gram-positive bacteria and that the closest neighbour of this organism (92.6–92.9 % similarity) was the cloned 16S rRNA gene sequence of a not-yet cultured bacterium, thought to represent *Oscillospira guilliermondii*. The nearest cultivated neighbours of strain Sjm18-20^T were *Clostridium orbiscindens* DSM 6740^T and *Clostridium viride* T2-7^T, with sequence similarities of 91.3 and 89.1 %, respectively. On the basis of phenotypic features and phylogenetic position, it is proposed that this isolate represents a novel species in a new genus, *Oscillibacter valericigenes* gen. nov., sp. nov. The type strain of *Oscillibacter valericigenes* is Sjm18-20^T (=NBRC 101213^T =DSM 18026^T).

Clostridial bacteria, which are mainly low-G+C-content Gram-positive, endospore-forming anaerobic bacteria, are extremely heterogeneous phylogenetically and form 19 clusters based on 16S rRNA gene sequence information (Collins *et al.*, 1994). Within the clostridial bacteria, clostridial cluster IV is also phenotypically heterogeneous and includes *Clostridium orbiscindens*, which is capable of cleaving the flavonoid C-ring (Winter *et al.*, 1991), *Clostridium viride*, which ferments 5-aminovaleric acid to ammonia, valeric acid, propionic acid and acetic acid (Buckel *et al.*, 1994), *Papillibacter cinnamivorans*, which transforms cinnamic acid to acetic acid and benzoic acid (Defnoun *et al.*, 2000), and *Sporobacter termitidis*, which is capable of cleaving the ring of methoxylated aromatic compounds (Grech-Mora *et al.*, 1996). The habitat of anaerobic bacteria belonging to clostridial cluster IV is the alimentary canal and faeces of various organisms such as humans and wood-feeding termites, anaerobic sewage sludge and anaerobic digesters.

Recognition of their diversity and development of cultivation methods for strictly anaerobic bacteria inhabiting the alimentary canals of various animals are thus important in understanding host adaptation to the degradation of cellulose and other alimentation.

In this paper, the isolation of a mesophilic, strictly anaerobic bacterium with a 16S rRNA gene sequence that is phylogenetically related to those of bacteria included in clostridial cluster IV is described. On the basis of morphological, biochemical, physiological and phylogenetic properties, a novel genus and species are proposed for this bacterium.

Japanese corbicula clams (*Corbicula japonica*) were collected on a sea coast in Shimane Prefecture in Japan. Samples collected were kept in a sealed nylon bag with an O_2-absorbing and CO_2-generating agent (Anaero-Pack; Mitsubishi Gas Chemical) during transfer to our laboratory.

Abbreviation: DMA, dimethylacetal.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Oscillibacter valericigenes* Sjm18-20^T is AB238598.

The basal medium was composed of 10 g yeast extract (Becton Dickinson), 5 g polypeptone (Nihon Pharmaceutical), 0.025 g Tween 80, 5 ml salts solution and 1 l distilled water. The pH of the medium was adjusted to 6.0. The salts solution contained (l^{-1} distilled water) 40 g $MgSO_4 . 7H_2O$, 2 g $MnSO_4 . 4H_2O$, 2 g $FeSO_4 . 7H_2O$ and 2 g NaCl. LYPm medium was prepared by adding 10 g α-lactose and 20 g NaCl to 1 l basal medium.

Several Japanese corbicula clams were dissected and approximately 1 g alimentary canal material was used for the isolation of bacteria. The alimentary canal material was minced mechanically and suspended in saline. Serial decimal dilutions (10^{-1} to 10^{-10}) of the suspension were made with saline and 0.1 ml diluted samples were spread on LYPm agar (1.5 % w/v) plates and cultivated at room temperature (approx. 20–25 °C) in a sealed nylon bag with an O_2-absorbing and CO_2-generating agent for at least 1 month. Visible colonies grown on LYPm agar medium were picked up and transferred to vials containing fresh LYPm medium in which air was replaced with nitrogen gas by flushing. The vials were subsequently sealed with tight-fitting butyl rubber stoppers and incubated at room temperature for 1 month. Cultures were further purified anaerobically on slants of LYPm medium solidified with 1.5 % (w/v) agar. The purification procedure was repeated several times until the cultures were deemed pure and a uniformly shaped axenic culture, designated Sjm18-20^T, was obtained. After purification, isolates were maintained in nitrogen-gas-flushed GYP medium (10 g D-glucose added to 1 l basal medium).

Cells of strain Sjm18-20^T were straight to slightly curved rods, approximately 0.4–0.6 × 2.5–6.0 μm in size (Fig. 1a). They had rounded ends and tapered to one pole. Oscillating motility was observed under the microscope. Electron microscopy demonstrated the presence of peritrichous flagella. Moreover, cells of strain Sjm18-20^T elongated in 3-month-old cultures, forming long rods of 0.5 × 10–35 μm (Fig. 1b). Ultrathin sections of whole cells of strain Sjm18-20^T revealed a cytoplasmic membrane surrounded by a surface layer (Fig. 1c, d). The Gram reaction of the cells was negative based on the Hucker–Conn method (Hucker & Conn, 1923). Spore formation was not observed even though cells from various stages of the growth phase were observed microscopically. In addition, the presence of spores was analysed by testing the heat resistance of cells in culture; however, there was no cell growth after heat treatment indicating the lack of heat-resistant bodies such as spores.

Strain Sjm18-20^T was strictly anaerobic and catalase-negative. The growth temperature for strain Sjm18-20^T was 15–35 °C, with optimum growth at 30 °C. No growth was observed at 10 or 40 °C. The isolate grew at pH 5.5–8.5, with optimum growth at pH 6.0–6.5. No growth was observed at pH 5.0 or 9.0. Growth occurred below 4.0 % (w/v) NaCl. No growth was observed in 6.0 % (w/v) NaCl. Strain Sjm18-20^T grew fermentatively and produced acids from D-glucose, L-arabinose, D-ribose and D-xylose. No growth occurred on D-arabinose, D-mannose, D-galactose, D-fructose, D-sorbose, L-rhamnose, maltose, D-cellobiose, D-melibiose, trehalose, α-lactose, sucrose, D-raffinose, D-melezitose, starch, D-sorbitol, D-mannitol, *myo*-inositol, D-salicin or sodium gluconate. *n*-Valeric acid was the major end product from glucose, as determined by HPLC equipped with an organic acid column (Waters). Elemental sulfur (1 %), sulfate (20 mM), thiosulfate (20 mM), sulfite (10 mM), nitrate (10 mM), nitrite (10 mM) and fumarate

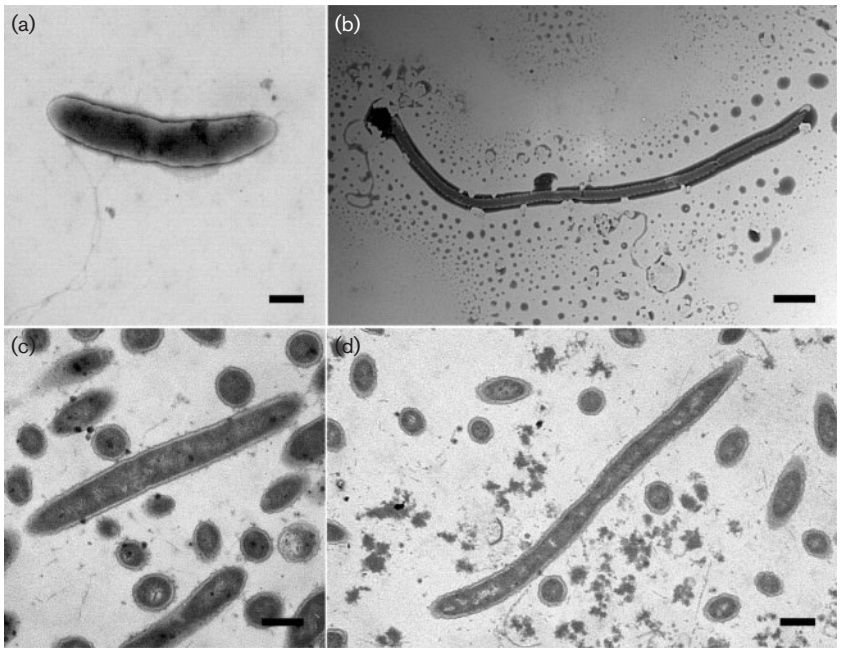

Fig. 1. Transmission electron micrographs of cells of strain Sjm18-20^T. (a, b) Negatively stained cells cultivated for 1 week (a) and 3 months (b). (c, d) Ultrathin sections of cells cultivated for 1 week (c) and 3 months (d). Bars, 0.5 μm (a, c, d) and 2.0 μm (b).

(20 mM) were not utilized as electron acceptors in the presence of yeast extract and polypeptone [0.5 % (w/v) of each] as carbon and energy sources. The generation time under optimum growth conditions was calculated to be 18.3 h, based on the increase in turbidity.

The major cellular fatty acids were iso-$C_{15:0}$ (13.4 %), $C_{14:0}$ (9.2 %), $C_{18:1}\omega9c$ (9.1 %) and anteiso-$C_{15:0}$ (6.7 %) using the MIDI microbial identification system (Microbial ID; Agilent Technologies) based on the method described by Komagata & Suzuki (1987). Fatty aldehydes were also found among the cellular fatty acid methyl esters as dimethylacetals (DMAs) such as $DMA_{16:0}$ (14.7 %), $DMA_{14:0}$ (10.4 %) and iso-$DMA_{15:0}$ (7.4 %). The genomic DNA G+C content of strain Sjm18-20^T was 52.9 mol%, determined by HPLC as described by Tamaoka & Komagata (1984).

An almost-complete 16S rRNA gene sequence (1453 bases) was determined for strain Sjm18-20^T. The 16S rRNA gene was amplified by PCR with primers U27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and U1492R (5′-GGTTACCTTGTTACGACTT-3′). After alignment with the ARB software (http://www.arb-home.de/), the phylogenetic tree was constructed by the neighbour-joining method with the program CLUSTAL_X (Felsenstein, 1985; Kimura, 1980; Saitou & Nei, 1987; Thompson *et al.*, 1997) and the maximum-likelihood method with MORPHY software (Adachi & Hasegawa, 1995). Phylogenetic analysis based on alignment of 1320 bp 16S rRNA gene sequences showed that strain Sjm18-20^T was part of clostridial cluster IV of the low-G+C-content Gram-positive bacteria as defined by Collins *et al.* (1994) (Fig. 2). The 16S rRNA gene sequence of strain Sjm18-20^T had sequence similarities of 92.6–92.9 % to cloned sequences from not-yet cultured cells (OSC1, OSC2, OSC3, OSC4 and OSC5), thought to represent *Oscillospira guilliermondii* Chatton and Pérard 1913 (Yanagita *et al.*, 2003; Mackie *et al.*, 2003). The nearest cultivated neighbours of this strain were *C. orbiscindens* DSM 6740^T and *C. viride* T2-7^T, with sequence similarities of 91.3 and 89.1 %, respectively. Strain Sjm18-20^T was distantly related to *S. termitidis* SYR^T and *P. cinnamivorans* CIN1^T, with respective sequence similarities of 88.7 and 88.6 %.

Morphological, biochemical and physiological properties of strain Sjm18-20^T and phylogenetically related strains are summarized in Table 1. Strain Sjm18-20^T could be differentiated from related cultivated strains, namely *C. orbiscindens* 265^T, *C. viride* T2-7^T, *S. termitidis* SYR^T and *P. cinnamivorans* CIN1^T, by morphological, biochemical and physiological properties. Cells of strain Sjm18-20^T were larger than those of *C. viride* T2-7^T, *S. termitidis* SYR^T and *P. cinnamivorans* CIN1^T. Moreover, two properties of strain Sjm18-20^T, non-sporulation and Gram-negative staining, significantly distinguished strain Sjm18-20^T from related cultivated strains. In addition, strain Sjm18-20^T produced acid from D-glucose and a few pentoses such as L-arabinose, D-ribose and D-xylose. This ability obviously distinguished strain Sjm18-20^T from other related strains in clostridial cluster IV. Strain Sjm18-20^T did not produce acid from the various hexoses tested except for D-glucose, disaccharides, oligosaccharides, polysaccharides or sugar alcohols.

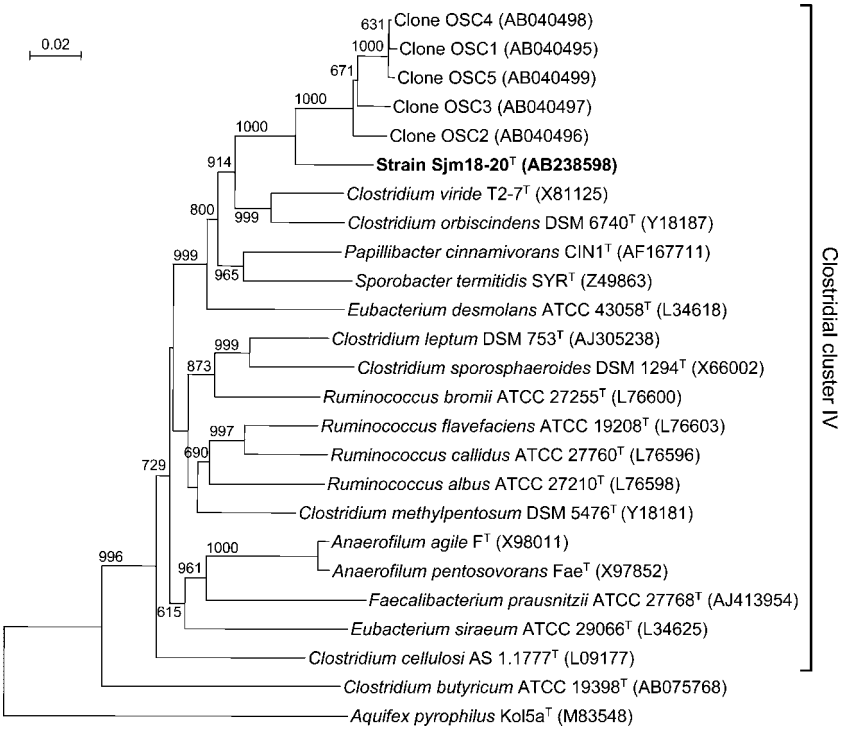

Fig. 2. Phylogenetic tree showing the relationship of strain Sjm18-20^T within clostridial cluster IV of the low-G+C-content Gram-positive bacteria. The tree was based on an alignment of 1320 bp 16S rRNA gene sequences and constructed by using the neighbour-joining method. Numbers at nodes indicate bootstrap values, derived from 1000 bootstrap replications. Bar, 0.02 substitutions per nucleotide position.

Table 1. Morphological, biochemical and physiological properties of strain Sjm18-20^T (*Oscillibacter valericigenes* gen. nov., sp. nov.) and its phylogenetic relatives

All taxa are strictly anaerobic. ND, No data available.

Character	Strain Sjm18-20^T	*C. orbiscindens* 265^T	*C. viride* T2-7^T	*S. termitidis* SYR^T	*P. cinnamivorans* CIN1^T	*Oscillospira guilliermondii*
Morphology	Straight or slightly curved rods	Straight rods	Oval rods	Slightly curved rods	Straight rods	Large rods, often curved
Cell size (μm)	0.5 × 2.0–5.0 (often 0.5 × 30)	0.9–1.0 × 2.0–7.0	0.8 × 1.2–1.5	0.2–0.4 × 1.0–2.0	0.5–0.6 × 1.3–3.0	3–6 × 10–40
Motility	Motile	Motile	Motile	Motile	Non-motile	Motile
Flagella	Peritrichous flagella	Peritrichous flagella	Two subpolarly inserted flagella	Peritrichous flagella	None	Peritrichous flagella
Spore formation	None	Spore-forming	None	Spore-forming	None	Endospore
Gram staining	Negative	Variable	Positive	Positive	Positive	Negative
Catalase reaction	Negative	ND	Negative	ND	ND	ND
Temperature for growth (°C):						
Optimum	30	37	ND	32–35	37	ND
Range	15–35	ND	19–40	20–40	15–40	ND
Initial pH for growth:						
Optimum	6.0–6.5	ND	ND	6.7–7.2	7.5	ND
Range	5.0–8.5	ND	ND	5.9–8.8	6.9–8.5	ND
NaCl requirement (%):						
Optimum	0	ND	ND	0–0.5	0.5–1.0	ND
Range	0–4	ND	ND	<1.25	<2.0	ND
Acid production from:						
L-Arabinose	+	−	−	ND	−	ND
D-Ribose	+	−	−	−	−	ND
D-Xylose	+	−	−	−	−	ND
D-Glucose	+	−	−	−	−	ND
DNA G+C content (mol%)	52.9	56–57	41.5	57	56	ND
Source	Alimentary canal of Japanese corbicula clams	Normal human faecal flora	Anaerobic sewage sludge	Hindgut of wood-feeding termite	Anaerobic digester feed	Alimentary canal of herbivorous animals

Phylogenetically, strain Sjm18-20 was located near the clade of *Oscillospira guilliermondii*-like cells that had been separated by flow cytometric sorting without cultivation (Yanagita *et al.*, 2003). *Oscillospira guilliermondii* is a large bacterium (3–6 × 10–40 μm) that exhibits oscillating motility and was first discovered from the caecal contents of a guinea pig in 1913 (Chatton & Pérard, 1913; Gibson, 1974). Currently, *Oscillospira guilliermondii* is the only species with a validly published name belonging to the genus *Oscillospira* (Skerman *et al.*, 1980; http://www.bacterio.cict.fr/), and it was listed as the only member of the family *Oscillospiraceae* by Gibson (1974). Cells of strain Sjm18-20^T were normally slightly curved rods, 2.5–6.0 μm long; they formed longer rods (up to 30 μm) after prolonged cultivation. Strain Sjm18-20^T was strictly anaerobic, Gram-negative and exhibited oscillating motility by means of peritrichous flagella. These properties are similar to those described for *Oscillospira guilliermondii* (Chatton & Pérard, 1913; Gibson, 1974). However, strain Sjm18-20^T did not form a multicellular structure or endospores, which are unique characteristics of *Oscillospira guilliermondii* (Chatton & Pérard, 1913). Thus, the morphological properties of strain Sjm18-20^T, although similar, differed from those of *Oscillospira guilliermondii*. However, it is difficult to compare characteristics of the two bacteria further as *Oscillospira guilliermondii* has not yet been isolated, despite being observed and described almost 100 years ago. As a result, definitive evidence could not be obtained as to whether or not strain Sjm18-20^T should be accommodated in the genus *Oscillospira*. It will be important to isolate *Oscillospira guilliermondii* strains that exhibit the morphological properties described by Chatton & Pérard (1913) to clarify the relationship between strain Sjm18-20^T and *Oscillospira guilliermondii*.

On the basis of its phylogenetic position, morphology and biochemical and physiological properties described above, strain Sjm18-20^T differs significantly from members of related cultivated genera, namely *Sporobacter* and *Papillibacter*, and other clostridial strains. Consequently, it is proposed that strain Sjm18-20^T represents a novel species in a new genus, *Oscillibacter valericigenes* gen. nov., sp. nov.

Description of *Oscillibacter* gen. nov.

Oscillibacter (Os.cil.li.bac′ter. L. n. *oscillum* a swing; N.L. masc. n. *bacter* rod; N.L. masc. n. *Oscillibacter* the oscillating rod).

Strictly anaerobic, mesophilic, neutrophilic, Gram-negative-staining, non-sporulating and motile by peritrichous flagella. Cells form straight to slightly curved rods and often form elongated rods after prolonged cultivation. Represents a distinct phylogenetic lineage in clostridial cluster IV of the low-G+C-content Gram-positive bacteria branch based on 16S rRNA gene sequence analysis. The type species is *Oscillibacter valericigenes*.

Description of *Oscillibacter valericigenes* sp. nov.

Oscillibacter valericigenes [va.le.ri.ci.ge′nes. N.L. n. *acidum valericum* valeric acid; N.L. suff. *-genes* (from Gr. v. *gennaô* to produce) producing; N.L. part. adj. *valericigenes* producing valeric acid].

Displays the following properties in addition to those given in the genus description. Cells are 0.5 × 2.5–6.0 μm in size, often 0.5 × 30 μm after prolonged cultivation. Grows at 15–35 °C and pH 5.5–8.5, with optimum growth at 30 °C and around pH 6.0–6.5. Growth occurs below 4.0 % (w/v) NaCl. Catalase-negative. Acids are produced from D-glucose, L-arabinose, D-ribose and D-xylose. No growth occurs with D-arabinose, D-mannose, D-galactose, D-fructose, D-sorbose, L-rhamnose, maltose, D-cellobiose, D-melibiose, trehalose, α-lactose, sucrose, D-raffinose, D-melezitose, starch, D-sorbitol, D-mannitol, *myo*-inositol, D-salicin or sodium gluconate. *n*-Valeric acid is the major end product from glucose. Sulfate, sulfite, thiosulfate, elemental sulfur, nitrate, nitrite and fumarate are not used as electron acceptors. The major cellular fatty acids and fatty aldehydes are $C_{14:0}$, $C_{18:1}\omega9c$, iso-$C_{15:0}$, anteiso-$C_{15:0}$, $DMA_{14:0}$, $DMA_{16:0}$ and iso-$DMA_{15:0}$.

The type strain is Sjm18-20^T (=NBRC 101213^T =DSM 18026^T), isolated from alimentary canal material from Japanese corbicula clams collected on the sea coast in Shimane, Japan. The genomic DNA G+C content of the type strain is 52.9 mol% (as determined by HPLC).

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