Caloramator viterbensis sp. nov., a novel thermophilic, glycerol-fermenting bacterium isolated from a hot spring in Italy

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A moderately thermophilic, anaerobic bacterium, strain JW/MS-V55T, was isolated from a mixed sediment/water sample of a hot spring at Bagnaccio (near Viterbo, Italy). The cells of this organism were straight to slightly curved rods, 0.4–0.6 × 2.0–3.0 µm in dimension. Cells occurred singly and stained Gram-positive. The temperature range for growth at pH25°C 6.0 was 33–64°C, the optimum being 58°C. The pH25°C range for growth was from 5.0 to 7.8, the optimum being 6.0–6.5. The substrates utilized included glycerol, glucose, fructose, mannose, galactose, sucrose, cellobiose, lactose, starch and yeast extract. Acetate and 1,3-propanediol were the only detectable organic products of glycerol fermentation; significant amounts of H2 were produced during growth. The strain was unable to grow autotrophically in the presence of H2 and CO2. The main products of glucose fermentation were CO2, H2, acetate and ethanol. Single amino acids, including serine, glutamine, threonine, leucine, methionine, aspartate, valine and histidine (but not arginine), served as carbon sources. Growth was completely inhibited by ampicillin, chloramphenicol, erythromycin, rifampicin and kanamycin at 100 µg ml⁻¹ and was retarded by streptomycin and tetracycline. The G+C content of the DNA was 32 mol% (HPLC). According to 16S rDNA sequence analysis, the isolate is located within the Gram-type positive Bacillus–Clostridium branch of the phylogenetic tree. On the basis of physiological properties and phylogenetic analysis, it is proposed that strain JW/MS-V55T (the only, and type, strain) (= DSM 13723T = ATCC PTA 5847), constitutes the new species Caloramator viterbensis.

Keywords: glycerol fermentation, 1,3-propanediol, Firmicutes, anaerobic, thermophiles

INTRODUCTION

Anaerobic breakdown of glycerol has been described for a variety of different micro-organisms. Despite its widespread distribution in nature (e.g. as a constituent of lipids and as an osmoregulant), relatively few micro-organisms are known to be able to grow anaerobically on glycerol in the absence of external electron acceptors. Compared to glucose, utilization of glycerol as a substrate requires disposal of additional reducing equivalents, because of its more reduced redox state. The extra reducing equivalents create a problem when oxygen, as a terminal electron acceptor, is not available. Different micro-organisms use different pathways to utilize glycerol under anaerobic conditions. One example is the reduction of acetyl CoA to ethanol as described for Klebsiella planticola (Jarvis et al., 1997). Electrons can also be disposed of by the release of molecular hydrogen. Anaerovibrio glycerini performs a propionic acid fermentation coupled with the production of molecular hydrogen during growth on glycerol (Schauder & Schink, 1989). The homoacetogenic Moorella glycerini employs the Wood–Ljungdahl pathway to reduce CO2, so that glycerol can be converted stoichiometrically to acetate (Slobodkin et al., 1997). Other acetogenic bacteria, however, such as Acetobacterium woodii and Acetobacterium carbinol-...
licum, and the majority of micro-organisms currently described as anaerobic glycerol-utilizers, form 1,3-propanediol as the major reduced fermentation product (Emde & Schink, 1987). The corresponding pathway involves a dismutation of glycerol, part of which is funnelled into the energy- and biomass-producing branch; the other part is converted reductively to 1,3-propanediol, thereby regenerating NAD\(^+\) (Abeles \textit{et al.}, 1960; Forage & Foster, 1982). This pathway has been demonstrated for bacteria originating from diverse phylogenetic groups (Stieb & Schink, 1984; Forsberg, 1987; Bouvet \textit{et al.}, 1995; Qatibi \textit{et al.}, 1998), but not previously for any thermophilic micro-organism.

In this paper, we describe an anaerobic, Gram-type positive (Wiegel, 1981), glycolytic and amino-acid-utilizing (eu)bacterial thermophile, \textit{Caloramator viterbensis} sp. nov., isolated from a hot spring at Viterbo (Italy), which utilizes glycerol as a growth substrate and converts it to 1,3-propanediol as the main fermentation product.

**METHODS**

**Source of organism.** The strain was isolated from a mixed sediment/water sample collected from a freshwater hot spring in the Bagnaccio Spring area near Viterbo, Italy, in June 1997. The temperature at the sampling points was 63 °C and the pH was 6.6–6.7 (for a detailed analysis, see Canganella & Trovatelli, 1995).
Media and cultivation. For enrichment, isolation and culturing, a basal medium was employed. It was prepared under a N₂/CO₂ (80:20) gas phase by using the modified Hungate technique (Ljungdahl & Wiegel, 1986) and contained the following (g per l denized water unless otherwise indicated): (NH₄)₂SO₄, 0.5; NH₄Cl, 0.5; KH₂PO₄, 20; MgCl₂, 6H₂O, 0.04; CaCl₂, 2H₂O, 0.04; NaHCO₃, 42; cysteine, HCl, 0.13; yeast extract, 0.3; resazurin, 0.001; glycerol, 3.0; vitamin solution (Wolin et al., 1963), 2 ml; and trace-element solution, 1 ml. The trace-element solution contained the following (mM): (NH₄)₆Fe(SO₄)₂, 6H₂O, 2.0; CoCl₂, 6H₂O, 10; (NH₄)₂Ni(SO₄)₂, 6H₂O, 10; Na₂MoO₄, 2H₂O, 0.1; NaWO₄, 2H₂O, 0.1; K₂ZnO₂, 7H₂O, 0.5; CuCl₂, 2H₂O, 0.01; Na₂SeO₃, 0.5; H₂BO₃, 0.1; MnCl₂, 4H₂O, 0.5; and Al₂(SO₄)₃·12H₂O, 0.01. The reducing agent was added to the medium, after boiling and degassing, from an anaerobic stock solution to a final concentration of 0.13 g Na₂S·9H₂O per l medium. The pH was adjusted to 6.0 (at 25°C) before autoclaving and was checked thereafter. Enrichments and pure cultures were grown usually in 10 ml medium in Hungate tubes under an atmosphere of N₂/CO₂ (80:20). All incubations were at 60°C unless indicated otherwise.

Determination of growth. Growth of bacteria was determined by measuring the increase in optical density at a wavelength of 600 nm (Spectronic 21; Bausch & Lomb). The absence of possibly interfering precipitation was checked by microscopy.

pH and temperature ranges. For the determination of the pH range for growth, the pH was determined at 25°C using a model 815 MP pH meter (Fisher Scientific). The temperature range for growth was determined using a temperature-gradient incubator (Scientific Industries), modified by the University of Georgia Institute Shop, under shaking (15 r.p.m.) in basal medium at pH6±0.6.

Substrate utilization. The ability of the micro-organism to utilize different substrates was tested by using the basal medium supplemented with 0.5% (w/v) filter-sterilized substrates instead of glycerol. The cultures were incubated for 2 weeks and monitored for growth by measuring the optical density and lowering the pH.

Electron acceptors. The potential use of different electron acceptors was tested in the basal medium containing glycerol (3 g l⁻¹) as the substrate. The different electron acceptors were added from autoclaved stock solutions. Cultures in the exponential growth phase growing in basal medium were used as the inocula (10% v/v). The use of the electron acceptors (10 mM) was monitored by determining nitrite production (for nitrate), sulfide production (for sulfate and elemental sulfur) or changes in colour (9,10-anthraquinone-2,6-disulfonic acid).

Antibiotic susceptibility. Susceptibility to antibiotics was determined by transferring an exponentially growing culture into fresh basal media containing filter-sterilized antibiotics at 100 μg ml⁻¹. The cultures were incubated for 2 weeks at 60°C.

Microscopy. Routine examinations were performed using light microscopy (model PM 10AD equipped with phase-contrast optics; Olympus). Transmission electron microscopy was performed with a JEOL 100CX electron microscope. The samples used for ultrathin sectioning were prepared by using uranyl acetate and lead citrate for poststaining, as described by Spurr (1969). Gram staining was performed using the Hucker method (Doetsch, 1981).

Analytical techniques. Determination of glycerol, glucose, short-chain organic acids and alcohols was performed by HPLC and molecular hydrogen was analyzed by GC, as described by Svetlitshnyi et al. (1996). Production of nitrite was measured using an enzymic analysis kit from Boehringer Mannheim (catalogue no. 905608). Sulphide was determined by the method of Cord-Ruwisch (1985).

G+C content of the DNA. The DNA was isolated and purified using the Qiagen genomic DNA-purification protocol according to the manufacturer’s instructions. The DNA was digested enzymically, and the G+C content was determined by separating the nucleosides by HPLC as described by Whitman et al. (1986) and Mesbah et al. (1989).

16S rRNA gene sequence determination and phylogenetic analyses. The extraction of genomic DNA, PCR amplification of the 16S rRNA gene, and sequencing of the purified PCR products were carried out as described previously (Rainey et al., 1996). Sequence reaction products were purified by ethanol precipitation and electrophoresed with a model 310 Genetic Analysrer (Applied Biosystems). The 16S rRNA gene sequences obtained in this study were aligned against the previously determined low-G+C, Gram-positive sequences available from the public databases by using the ae2 editor (Maidak et al., 1999). The programs of the PHYLIP package, including DNADIST and NEIGHBOR, were used for the phylogenetic analyses (Felsenstein, 1993). The method of Jukes & Cantor (1969) was used to calculate evolutionary distances. The tree topology was reanalysed using 1000 bootstrapped datasets and the programs SEQBOOT, DNADIST and CONSENSE of the PHYLIP package (Felsenstein, 1993).

RESULTS

Enrichment and isolation

The basal medium containing glycerol was inoculated with approximately 10% (w/v) of the sample and was incubated at 60°C. In addition, media of the same composition exhibiting pH values of either 7.5 or 9.0 were inoculated with the sample (10%, w/v, each). Only at pH 6.0 was an enrichment culture obtained that utilized glycerol. After subsequent transfers (10%, v/v), dilution series of this enrichment culture were prepared and plated on solid basal medium (1.5% agar), using soft agar overlays that consisted of basal medium containing 0.8% agar. Single colonies were picked from the anaerobic chamber and suspended in about 0.5 ml anaerobic medium. About 0.2 ml was used to subculture the cells in liquid medium of the same composition and to determine the formation of 1,3-propanediol by HPLC. The other 0.2 ml was used for subsequent plating. The procedure was repeated five times using basal medium as well as a defined complex medium described by Kell et al. (1981) but which was adjusted with 2 M HCl to pH 6.0 and supplemented with 3 g glycerol l⁻¹. Growth in liquid medium was largely enhanced when the cultures were incubated with shaking and the basal medium was supplemented with yeast extract to a final concentration of 2 g l⁻¹. After transfer of the colonies into liquid basal medium at 60°C with shaking, a glycerol-fermenting culture was obtained which was considered as pure; this was designated as strain JW/MS-VSS5T.
Colony and cell morphology

On plates containing basal medium, the colonies appeared after 7–10 days. Once the micro-organism had adapted to growth on plates, subsequent streaks on fresh plates yielded colonies after 2–3 days. The colonies were uniformly round, white and 1.0–1.5 mm in diameter. Cells of strain JW/MS-V5T grown in liquid cultures under agitation were straight to slightly curved rods 0.4–0.6 μm in diameter and 2.0–3.0 μm in length (Fig. 1a). The cells mostly occurred singly. Growth in liquid cultures without agitation predominantly yielded longer cells and cell aggregates (Fig. 1b) with the tendency to flocculate. Cells in the mid- and late-exponential growth phase frequently exhibited swollen ends (reminiscent of prespores) and bulging sections in the middles of elongated cells (Fig. 1c–g). No indication of motility was obtained by using light microscopy. Electron microscopy analysis performed after negative staining did not reveal the presence of flagella among cells from different growth stages. The occurrence of spores was not observed by microscopy or by heat resistance (10 min at 100 °C) of cultures grown to the stationary growth phase.

Gram-staining reaction and Gram type

The cells stained Gram-positive only in the early exponential growth phase. Ultrathin sections of strain JW/MS-V5T (Fig. 2) revealed a thick peptidoglycan layer; thus, the organism was regarded as Gram-type positive (Wiegel, 1981). This is consistent with the 16S rRNA sequencing data, which placed the organism in the Clostridium–Bacillus branch of the phylogenetic tree. In addition to the peptidoglycan layer, another outer layer was observed (Fig. 2), which presumably represents an outer protein (S-)layer; however electron micrographs failed to reveal any geometrical arrays.

Temperature and pH ranges

The temperature range, at pH 6.0, for growth of strain JW/MS-V5T was 33–64 °C, the optimum being 58 °C (Fig. 3). No growth was detected at 67 °C or at temperatures lower than 33 °C. At 60 °C, the strain grew at pH 6.0 range 5.0–7.8, the optimum at pH 6.8 being 6.0–6.5 (Fig. 4). No growth was detected at pH 4.7 or pH 8.0. The shortest doubling time under optimal growth conditions was 2.8 h.

Substrate utilization and fermentation products

The substrates utilized included glycerol, glucose, fructose, sucrose, cellobiose, lactose, galactose, mannose (20 mM), starch and yeast extract (5 g l⁻¹). The amino acids (0.5%, w/v) serine, glutamine, threonine,
leucine, methionine, aspartate, histidine and valine, but, surprisingly, not arginine, were used as carbon and nitrogen sources when added to the basal medium lacking glycerol. Strain JW/MS-VS5T did not use xylose, arabinose, acetate, lactate, formate, methanol, ethanol, n-propanol, iso-propanol, n-butanol, propionate, acetone, succinate, ethylene glycol, 1,2-propanediol, phenol, benzoate or H$_2$/CO$_2$. Fermentation of glycerol yielded acetate and 1,3-propanediol as the only organic metabolic products (Table 1). No C$_1$–C$_3$ alcohols, diols other than 1,3-propanediol, or organic acids other than acetate were detected in measurable amounts. Significant amounts of H$_2$ were produced in these cultures. The fermentation pattern obtained suggests a conversion of glycerol according to the following equation:

$$3 \text{Glycerol} \rightarrow 2 \text{1,3-propanediol} + \text{acetate} + \text{CO}_2 + \text{H}_2.$$ 

Glucose was fermented mainly to ethanol, CO$_2$, H$_2$ and acetate at the approximate ratio 1:1:2:0.4:0.2.

Electron acceptors

In the presence of glycerol as a substrate, strain JW/MS-VS5T did not reduce nitrate (10 mM), amorphous Fe(III) oxide (90 mM), 9,10-anthraquinone-2,6-disulfonic acid, sulfate (10 mM), or precipitated or sublimated S$_8$ (30 mM). Production of 1,3-propanediol was not affected in the presence of any of these electron acceptors. Strain JW/MS-VS5T was not capable of growth with O$_2$ (20%, v/v) in the gas phase. However, the effect of maintaining a low H$_2$ partial pressure (by using mixed cultures containing hydrogen-utilizing micro-organisms) on the propandiol formation was not tested.

Antibiotic susceptibility

Ampicillin, chloramphenicol, erythromycin, rifampicin and kanamycin completely inhibited growth at a concentration of 100 µg antibiotic (ml medium)$^{-1}$. The addition of streptomycin or tetracycline at the same concentration resulted in retardation of growth.

DNA base composition

The G + C content of the genomic DNA was 32 mol% (HPLC).

16S rRNA gene sequence comparison

An almost complete 16S rRNA gene sequence of strain JW/MS-VS5T comprising 1480 nucleotides was determined. Two datasets were used for the phylogenetic analyses. Dataset 1 contained the sequence determined in this study and a selection of reference sequences from the low-G+C Gram-type positive bacteria, while dataset 2 contained the new sequence and the sequences available in the databases for the five species of the genera Caloramator and Thermobrachium. Phylogenetic analyses based on dataset 1 comprising 1206 unambiguous nucleotides between positions 98 and 1469 (Escherichia coli positions; Brosius et al., 1978) showed the new isolate to cluster together as a distinct lineage within the radiation of the previously described genera Caloramator and Thermobrachium (Fig. 5). Strain JW/MS-VS5T shows 91.7–94.1% 16S rRNA gene sequence similarity with species of the previously described genera Caloramator and Thermobrachium and shows 83.4–87.1% similarity to the sequences of the other taxa included in dataset 1 and shown in Fig. 5. The second dataset containing the sequences of the new isolate and those of the five other Caloramator and Thermobrachium species and comprising 1393 unambiguous nucleotides between positions 38 and 1469 (E. coli positions; Brosius et al., 1978) was used to calculate pairwise similarity values between the taxa of this cluster. The 16S rRNA gene sequence similarities between strain JW/MS-VS5T and the previously described taxa were in the range 91.3–93.7%. Within the Caloramator/Thermobrachium cluster the highest sequence similarity value (93.7%) was found between strain JW/MS-VS5T and Thermobrachium celere. Bootstrap analyses on the branches recovered in Fig. 5 clearly give support to the Caloramator/Thermobrachium cluster and the relationship between this group and the genus Clostridium cluster I as defined by Collins et al. (1994).

Maintenance

Cultures of strain JW/MS-VS5T can be kept for several days in the modified Kell et al. (1981) medium (see Enrichment and isolation) at room temperature or preferably at 4 °C. For long-term storage at −75 °C, cultures should be grown to mid- to late-exponential growth phase in modified Kell et al. (1981) medium containing 2% yeast extract and 3 g glycerol l$^{-1}$. The
culture, having been grown, is supplemented with 10% glycerol and 10% DMSO (both v/v) and kept for 15 min at room temperature before being frozen. Cultures have been kept viable under these conditions for more than 28 months. Testing for 1,3-propanediol formation after the cultures have been revived, however, must be done with subcultures grown in basal medium (preferably containing 2 g yeast extract l\(^{-1}\)).

**DISCUSSION**

The strain described, JW/MS-VS5\(^T\), is a moderately thermophilic, anaerobic (eu)bacterium that was isolated on the basis of its ability to ferment glycerol to 1,3-propanediol, a property which has not been reported for any thermophilic micro-organism so far. As is apparent from the phylogenetic analysis (Fig. 5), the strain is most closely related to *Thermobrachium celere* and four *Caloramator* species that form a distinct cluster within the Gram-type positive *Bacillus–Clostridium* branch of the phylogenetic tree. These five species are thermophilic, chemoorganoheterotrophic, anaerobic bacteria exhibiting a low G+C content, which also applies to strain JW/MS-VS5\(^T\). Table 2 shows a comparison of the morphological and physiological traits of five of the six species found in this cluster (detailed data for *Caloramator coolhaasii* unpublished). The formation of thermoresistant endospores has been demonstrated only for two *Caloramator* species (*Caloramator fervidus* and *Caloramator proteoclasticus*), but not for *Caloramator indicus*, *Thermobrachium celere* or isolate JW/MS-VS5\(^T\). However, the presence of three representative sporulation genes in *Thermobrachium celere* has been demonstrated, which indicates that this species is not asporogenic but non-sporulating (Brill, 1997; Brill & Wiegel, 1997).

Table 2. Selected characteristics of strain JW/MS-VS5\(^T\) (this study) and other bacteria

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2(^a)</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spores or heat-resistant cells</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Temperature range (°C)</td>
<td>33–64</td>
<td>&gt;37 to &lt;75</td>
<td>&gt;37 to &lt;75</td>
<td>&gt;37 to &lt;80</td>
<td>30 to &lt;68</td>
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<td>(&lt;70–80)</td>
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<td>(37–45) to</td>
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<tr>
<td>Temperature optimum (°C)</td>
<td>58</td>
<td>60–65</td>
<td>60–65</td>
<td>68</td>
<td>55</td>
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<tr>
<td>pH optimum</td>
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<td>8.0–8.5</td>
<td>8.1</td>
<td>7.0–7.5</td>
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<tr>
<td>Utilization of glycerol</td>
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<td>–</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Shortest doubling time (h)</td>
<td>2.80</td>
<td>0.17</td>
<td>0.33</td>
<td>0.75</td>
<td>0.50</td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>32</td>
<td>30–31</td>
<td>25</td>
<td>39</td>
<td>31</td>
</tr>
</tbody>
</table>

NR, Not reported.

* Different strains.
1997). Non-sporeforming bacteria were defined as bacteria which have all, or most, of the sporulation genes, but in which one or more gene products required for sporulation are missing or are non-functional. The observation of spore formation is no longer regarded as a systematically differential property (see p. 109 of Wiegel, 1982).

The G+C content determined for JW/MS-VSST is in the same range as that of Thermobrachium celere and Caloramator proteoclasticus (30–32 mol%), whereas the corresponding values reported for Caloramator indicus and Caloramator fervidus differ significantly from those determined for the other three species (25 and 39 mol%, respectively), which might be due to the use of different methods for determination.

The temperature range for growth of strain JW/MS-VSST is more similar to that of Caloramator proteoclasticus than it is to that of Thermobrachium celere or those of the other two Caloramator species. As they exhibit an optimum temperature below 60 °C, JW/MS-VSST and Caloramator proteoclasticus are considered as moderately thermophilic, whereas the optimum temperatures for the other three species are at or above 60 °C.

Despite its relatedness to Thermobrachium celere and the three Caloramator species, and the overall similarity with regard to their physiological properties, strain JW/MS-VSST shows certain characteristics that differ significantly from those reported for the other species found within this cluster. The pH optimum for growth of JW/MS-VSST (pH 6.0–6.5) is at least 1 pH unit lower than the values determined for the other species, e.g. strains of the alkalitolerant species Thermobrachium celere exhibit pH optima of 6.0–8.5.

The most striking difference is the doubling time for growth under optimum conditions. Values determined for isolate JW/MS-VSST under optimum conditions with either glycerol or other substrates (data not shown) were found to be close to 3 h, which is several times higher than the doubling times reported for the other members of this cluster (values range from 10 to 45 min). The conversion of glycerol to 1,3-propanediol has been demonstrated only for strain JW/MS-VSST. Thermobrachium celere does not use glycerol as a substrate at all. The difference in physiological properties parallels the rather low level of sequence similarity for 16S rDNA when isolate JW/MS-VSST is compared with its closest relatives (< 95% similarity), which suggests that this strain should be classified as a member of a separate species, designated as Caloramator viterbensis sp. nov., strain JW/MS-VSST representing the type strain of this species.

Description of Caloramator viterbensis sp. nov.

Caloramator viterbensis [vi.ter.ben/sis. L. masc. adj. viterbensis pertaining to Viterbo in Italy (ancient name Viterium), the region from which the strain was isolated].

Cells are straight to slightly curved rods 0.4–0.6 x 2.0–3.0 μm in dimension. Cells occur singly and stain Gram-positive. Neither spores, heat-resistant (85 °C for 15 min) cells nor motility have been observed. The temperature range for growth at pH 6.0 is 33–64 °C, the optimum at 58 °C. The pH50% range for growth is 5.0–7.8, with an optimum at 6.0–6.5. Growth is observed with glycerol, glucose, fructose, mannose, galactose, sucrose, cellobiose, lactose, starch and yeast extract. Xylose, arabinose, acetate, lactate, formate, methanol, ethanol, n-propanol, iso-propanol, n-butanol, propionate, acetone, succinate, ethylene glycol, 1,2-propanediol, phenol and benzoate are not fermented. Various single amino acids (0.5%, w/v), with the exception of arginine, serve as carbon and nitrogen sources. No growth occurs under autotrophic conditions in the presence of H2/CO2. Fermentation of glycerol yields acetate and 1,3-propanediol as the only detectable organic products; significant amounts of H2 are produced during growth. Glucose is mainly fermented to ethanol plus CO2, H2 and acetate. Growth is inhibited by ampicillin, chloramphenicol, erythromycin, rifampicin and kanamycin (100 μg ml–1). Addition of streptomycin and tetracycline of the same concentration results in retardation of growth. The G+C content of the DNA is 32 mol% (HPLC). Isolated from a hot spring at Bagnaccio (near Viterbo, Italy). The type strain is JW/MS-VSST (= DSM 13723T = ATCC PTA 584T).

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