**Sporomusa silvacetica** sp. nov., an Acetogenic Bacterium Isolated from Aggregated Forest Soil

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*Sporomusa silvacetica* sp. nov. **DG-1** (T = type strain) was isolated from well-drained, aggregated forest soil (**pH 6.0**) in east-central Germany. The cells were obligately anaerobic, slightly curved rods and were motile by means of laterally inserted flagella on the concave side of each cell. Typical cells were approximately 3.5 by 0.7 μm. Cells stained weakly gram positive, but thin sections revealed a complex multilayer cell wall. Spores were spherical and distended the sporangia. Growth and substrate utilization occurred with furulate, vanillate, fructose, betaine, fumarate, 2,3-butanediol, pyruvate, lactate, glycerol, ethanol, methanol, formate, and H₂-CO₂. With most substrates, acetate was the primary reduced product and was produced in stoichiometries indicative of an acetyl-coenzyme A pathway-dependent metabolism. Fumarate was dismutated to succinate and acetate. Methoxy and acrylate groups of various aromatic compounds were oxidized and reduced, respectively. Yeast extract was not required for growth. Cells grew optimally at approximately 30°C and **pH 6.8**; under these conditions and with fructose as the substrate, the doubling time was approximately 14 h. The lowest temperature that supported growth was between 5 and 10°C. The carbon monoxide dehydrogenase and hydrogenase activities were approximately 9 and 102 pmol min⁻¹ mg of protein⁻¹, respectively. A type β cytochrome was detected in the membrane. The G+C content was approximately 43 mol%. Phylogenetic analysis of the 16S ribosomal DNA indicated that **DG-1** was most closely related to members of the genus *Sporomusa* in the *Clostridium* subphylum of the gram-positive bacteria.

Low-molecular-weight aliphatic organic acids are present in mineral forest soil solutions and are believed to play roles in soil formation, solubility of toxic metals, and plant growth (23, 24, 30, 49, 58, 59). In this regard, acetate is a dominant organic acid detected in mineral soils (59), and it has been proposed that the acetate in mineral soils is produced primarily through the collective action of facultatively and obligately anaerobic microorganisms (35, 63). Although well-drained soils are not considered typical acetogenic habitats, supplementation of forest (34, 35), prairie (63), and tundra (46) soils with H₂ or CO results in the utilization of substrates and the production of acetate in stoichiometries approximating those expected for H₂- or CO-dependent acetogenesis. In addition, acetogenic consortia are readily enriched from mineral forest soils (35, 48, and leaf litter (36). To further evaluate the occurrence of acetogens in well-drained, aggregated soils, an acetogen was isolated from a beech forest in east-central Germany. The collective characteristics of this isolate (strain **DG-1** [T = type strain]) are not consistent with the characteristics of any previously described acetogenic bacterium, and it is proposed that this organism should be placed in a new species, *Sporomusa silvacetica*.

**MATERIALS AND METHODS**

**Soil collection.** Forest soil (a silty loam) was obtained from the mineral (Ah) horizon of a slightly acidic beech site in the Geisberger Forest in east-central Germany. The soil had a pH of approximately 6, a dry weight of 60%, and an organic carbon content of 80 g kg⁻¹ (dry weight) (see reference 35 for additional site and soil characteristics). The soil was collected in sterile glass containers and was utilized immediately after transportation to the laboratory.

**Enrichment cultures.** Soil samples were placed in an anaerobic chamber (100% N₂ gas phase; room temperature; Mecaplex, Grenchen, Switzerland) and added to anaerobic medium (approximately 1 g [wet weight] of soil per 10 ml of medium). The medium was then supplemented with either H₂-CO₂ (80:20) or a combination of vanillate (5 mM) and CO (100%) (the gas volumes were 20 ml per 120-ml vial or 10 ml per 30-ml vial). Enrichment cultures were incubated at 15°C, and stable enrichment cultures were obtained by repeated transfer in the same medium. After several transfers, enrichment cultures were streaked onto solid media (media supplemented with 1.5% agar). Isolated colonies were transferred to liquid media and assayed for substrate utilization and product formation.

**Composition of media and growth conditions.** Media were prepared anaerobically (31). Medium A was an undefined, carbonate-buffered medium (16). Medium B was medium A without yeast extract or resazurin. These media were supplemented under CO₂ into 120-ml serum vials (50 ml of medium per vial) or 27-ml culture tubes (7 ml of medium per tube), which were then crimp sealed and autoclaved. Medium C was a defined, carbonate-buffered medium containing trace element solution (ST10) (64, 65). This medium was adjusted to pH 6.5 prior to autoclaving, and an alternative vitamin solution (67) was utilized (1 ml liter⁻¹); after autoclaving, the medium was dispensed under N₂-CO₂ (90:10). Leaf leachate medium was prepared by incubating leaves (500 g) from the litter layer at Geisberg, Germany, in distilled water (750 ml) for 1 week at 20°C. The liquid was then decanted, centrifuged (10,000 × g, 20 min), filter sterilized, and made anoxic by gassing with 100% argon. The leaf leachate (pH 5.9) was diluted 1:1 with distilled water and was added to sterile 30-ml serum vials (10 ml per vial) under 100% argon. Soil extract medium was prepared by adding 20 g of soil to 75 ml of distilled, anoxic water. The resulting soil suspensions were placed on an end-over-end shaker (40 cycles min⁻¹) for 2 h at 4°C. The aqueous phase was then centrifuged (10,000 × g, 20 min), filter sterilized, and gassed with and dispensed under 100% argon.

The reduction of sulfate was determined by using a sulfate-enriched lactate medium (50) supplemented with 0.1 g of cysteine-HCl liter⁻¹; tubes were visually inspected for sulfide production (i.e., blackening of the precipitate). The reduction of nitrate was determined by using medium A supplemented with 5 mM KNO₃. The fixation of N₂ was determined by using a medium designed to assay N₂-fixing microorganisms (52); a trace element solution (T) (1 ml liter⁻¹) replaced yeast extract, and the medium was prepared with a 100% N₂ gas phase. Unless otherwise indicated, the temperature of incubation was 25°C.

**Transmission electron microscopy.** Cells were cultivated at 30°C in Medium A supplemented with 10 mM fructose and harvested by centrifugation. The cells were negatively stained with uranyl acetate (62). For thin-section preparations, cells were fixed in glutaraldehyde-OsO₄ and prepared by a standard protocol (60). Thin sections were stained with uranyl acetate and lead acetate (53). Specimens were observed with a model CEM 902A microscope (Zeiss, Oberkochen, Germany).

**Preparation of cell extract and enzyme assays.** Cells were cultivated in Medium A supplemented with 5 mM fructose. Cultures (total volume, 1.3 liters) were dispensed into centrifuge bottles in a Mecaplex anaerobic chamber (100% N₂ gas phase; room temperature) and centrifuged (10,000 × g, 20 min, 4°C). The
FIG. 1. Transmission electron micrographs of strain DG-1T. (A) Negatively stained preparation. The arrowheads indicate insertion points of flagella. (B) Thin section. Abbreviations: CM, cytoplasmic membrane; M, murein layer; OM, outer membrane.

cell pellet was washed three times with sodium phosphate buffer (50 mM, pH 7). The cell pellet was resuspended in 2 volumes of freshly prepared, anoxic lysozyme buffer (39), incubated for 1 h at room temperature, and centrifuged at 10,000 x g for 20 min at 4°C. The supernatant fluid was transferred to a serum vial (100% N\textsubscript{2} gas phase) and assayed immediately. Hydrogenase and carbon monoxide dehydrogenase activities were assayed at 30°C with Tris-hydrochloride (100 mM, pH 8.5)-benzylic viologen (1 mM)-dithiothreitol (1 mM) in the presence of H\textsubscript{2} (100%) or CO (100%) (19). Polyacrylamide gel electrophoresis (19) was performed in a temperature-controlled Mecaplex anaerobic chamber (100% N\textsubscript{2} gas phase) at 10°C, and in situ staining of gels (19) for carbon monoxide
dehydrogenase activity was performed at room temperature. Reduction of acetylene was used to evaluate nitrogenase activity (37).

Membrane preparation and redox difference spectra. Cells were cultivated in medium A supplemented with 10 mM fructose. Membranes were prepared from cell extracts by ultracentrifugation under aerobic conditions (26). Washed membranes were reduced with sodium dithionite, and reduced-minus-oxidized spectra were obtained with a model Uvikon 930 double-beam recording spectrophotometer (Kontron Instruments, Milan, Italy) at room temperature (26).

G+C content. Cells were washed with phosphate buffer (50 mM, pH 7.0), and DNA was extracted by the NaOH method (3). The G+C content was determined by high-performance liquid chromatography (42).

Analysis of 16S rDNA. Approximately 95% of the 16S rRNA gene (rDNA) sequence of strain DG-1T was determined by directly sequencing PCR-amplified 16S rDNA. The analysis was performed by workers at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA, and purification of the PCR products were performed by using previously described protocols (31). Purified PCR products were sequenced by using an ABI PRISM Ready Reaction dye terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.). Sequence reaction mixtures were electrophoresed with an Applied Biosystems model 373A DNA sequencer. The sequence data were put into alignment editor ae2 (40), aligned manually, and compared with available 16S rDNA sequences of representative organisms belonging to the Clostridium subphylum of the gram-positive bacteria. A dendrogram was constructed by using standard phylogenetic methods and the PHYLIP package (22, 32, 54).

Additional analytical methods. Growth and cell dry weight were determined as previously described (16). For fructose-containing cultures, a culture optical density at 660 nm of 1 corresponded to 133 mg (dry weight) of cells liter⁻¹. Protein content was determined by the Bradford method (6).

The amounts of substrates and products present in culture fluids and gas phases were determined by high-performance liquid chromatography and gas chromatography (16, 27, 35, 41). Soil pH was determined by using 1:2.5 suspensions of soil in 0.02 N CaCl₂, and soil dry weight was obtained by weighing samples before and after drying at 105°C for 16 h. The amount of total carbon in oven-dried (65°C), homogenized organic matter was determined with an element analyzer (CHN-O-Rapid instrument; Foss-Heraeus, Hanau, Germany). The nitrate content was determined colorimetrically (11). The results and values given below are representative of the results and values obtained in duplicate experiments.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Maximum optical density at 660 nm</th>
<th>Amt of product (mM)</th>
<th>Acetate⁴</th>
<th>Protocatechuate⁴</th>
<th>Acetate/substrate ratio⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>0.46</td>
<td>4.8</td>
<td>13.8</td>
<td>NA</td>
<td>2.88 (3.0)</td>
</tr>
<tr>
<td>Vanillate</td>
<td>0.11</td>
<td>6.5</td>
<td>4.5</td>
<td>6.2</td>
<td>0.69 (0.75)</td>
</tr>
<tr>
<td>H₂</td>
<td>0.08</td>
<td>23.3</td>
<td>6.6</td>
<td>NA</td>
<td>0.28 (0.25)</td>
</tr>
</tbody>
</table>

⁴ Values were corrected for the amount of acetate formed in controls lacking additional substrates.

⁵ The values in parentheses are the theoretical ratios expected for acetogenic consumption of substrates (20).

⁶ NA, not applicable.
Isolate DG-1T was obtained from a stable medium (medium B or C), soil extract, and leaf leachate media supplemented with H₂-CO₂. DG-1T produced acetate as the sole soluble end product from utilization of H₂. The purity of isolate DG-1T was ensured by restreaking an isolated colony three times. Cultures of DG-1T were considered to be pure based on uniform colony and cellular morphologies.

**Isolation.** Isolate DG-1T was obtained from a stable medium B enrichment culture containing H₂-CO₂. DG-1T produced acetate as the sole soluble end product from utilization of H₂. The purity of isolate DG-1T was ensured by restreaking an isolated colony three times. Cultures of DG-1T were considered to be pure based on uniform colony and cellular morphologies.

**Morphology.** Isolate DG-1T was a strictly anaerobic, motile, rod-shaped organism. Its cells were approximately 3.5 by 0.7 μm and slightly curved (Fig. 1). Flagellar staining (4) and electron microscopy revealed laterally inserted flagella on the concave side of each cell (Fig. 1A). Cells exhibited both tumbling motility and short, directional motility in wet mounts. Although Gram staining (13) and the KOH test (28) indicated that DG-1T was weakly gram positive, thin sections revealed a more complex, multilayer cell wall (Fig. 1B, inset). Small inclusion bodies were observed in thin sections. With fructose as the substrate, cells occasionally swelled and became teardrop shaped after growth began; terminal, spherical spores were observed in wet mounts prepared from old cultures. Free spores were rarely observed, and the capacity to sporulate appeared to decrease with prolonged laboratory cultivation. Colonies on solidified medium A supplemented with fructose were shiny, beige to slightly yellow, and 2 to 3 mm in diameter. The colony form was irregular with entire or slightly undulate margins.

**Temperature and pH optima.** Isolate DG-1T grew at temperatures ranging from 10 to 35°C (Fig. 2A); no growth was observed at 5 or 42°C during incubation for 1 month. The optimal temperature was 25 to 30°C. Growth was observed after a sporulated culture was heated for 10 min at 80°C. Growth was relatively rapid when the initial pH was between 5.5 and 7.7 (Fig. 2B).

**Doubling time.** In fructose-supplemented medium A (pH 6.8), the doubling times at 30 and 10°C were approximately 14 and 80 h, respectively.

**Fermentation stoichiometries.** Strain DG-1T produced acetate concomitantly with growth on fructose, vanillate, or H₂-CO₂ (data not shown). The substrate-product stoichiometries obtained from such cultures indicated that acetogenic consumption of substrates occurred (Table 1). The growth yield for cells grown in medium A supplemented with 5 mM fructose was approximately 61 mg (dry weight) of cells liter⁻¹, and 13.8 mM acetate was formed, yielding an acetate/biomass ratio of 226 mmol of acetate g (dry weight) of cells⁻¹. Protocatechuate was produced in near stoichiometry with the amount of vanillate consumed, indicating that the aromatic ring was not subject to breakage or to further transformation.

**Additional physiological characteristics.** In medium A, growth and substrate utilization were observed with the following substrates: ferulate, vanillate, fructose, betaine, fumarate, 2,3-butanediol, pyruvate, lactate, glycerol, ethanol, methanol, formate, and H₂-CO₂. In all cases, the substrate was converted to acetate. Protocatechuate and hydroferulate were additional products observed with vanillate and ferulate, respectively. H₂ and the O-methyl group of vanillate were utilized as cosubstrates. Fumarate was dismutated to succinate and acetate. Fumarate is also dismutated by *Clostridium acetobutylicum* and *Clostridium formicoaceticum* (18, 41). H₂ was not produced. Traces of methane were produced in addition to acetate in cultures grown with H₂-CO₂; production of trace levels of methane has also been observed with the acetogens *Clostridium thermoautotrophicum* (55), *Acetobacterium woodii* (10), and *Acetobacterium carbinolicum* (10).
repeatedly in sodium-deficient medium B (prepared by using potassium salts rather than sodium salts) supplemented with either fructose or H₂-vanillate (data not shown).

Nitrate, rather than CO₃, is the preferred terminal electron acceptor for Clostridium thermoautothrophicum and C. thermoautothrophicum and inhibits the ability of these acetogens to form acetate by repressing the electron transport system normally engaged in the acetyl coenzyme A (acetyl-CoA) pathway (21, 26, 57). When strain DG-1 was grown in the presence of nitrate, nitrate was not appreciably utilized and acetate production was not appreciably affected, indicating that nitrate was not used as an alternative electron acceptor by DG-1. Sulfate was also not used as an alternative electron acceptor. Strain DG-1 did not fix N₂.

**Enzyme activities and membrane spectrum.** The carbon monoxide dehydrogenase and hydrogenase activities in cell extracts were approximately 9 and 102 pmol min⁻¹ mg of protein⁻¹, respectively. Two electrophoretically distinct carbon monoxide dehydrogenase bands were observed in sodium-deficient medium B (prepared by using acetate by repressing the electron transport system normally engaged in the acetyl coenzyme A (acetyl-CoA) pathway (21, 26, 57). When strain DG-1 was grown in the presence of nitrate, nitrate was not appreciably utilized and acetate production was not appreciably affected, indicating that nitrate was not used as an alternative electron acceptor by DG-1. Sulfate was also not used as an alternative electron acceptor. Strain DG-1 did not fix N₂.

**G+C content and phylogenetic analysis of 16S rDNA.** The DNA base composition of DG-1 (Fig. 4). A similarity value of 97.2% was obtained when the 16s rDNA sequence indicated that DG-1 is closely related to the genus Sporomusa (35). The cytoplasmic fraction had absorption maxima at 431 and 561 nm (Fig. 3B), indicating that the cytoplasmic fraction was approximately 9 and 102 pmol min⁻¹ mg of protein⁻¹.

**FIG. 3**. The cytoplasmic fraction had absorption maxima at 431 and 561 nm (Fig. 3B), indicating that the cytoplasmic fraction was approximately 9 and 102 pmol min⁻¹ mg of protein⁻¹.

**DISCUSSION**

Its cell shape, Gram reaction, formation of spores, and lack of sulfate reduction indicated that strain DG-1 is closely related to the genus Clostridium. However, thin sections revealed a multilayered cell wall characteristic of gram-negative bacteria. The phylogenetic analysis (Fig. 4) demonstrated that DG-1 is closely related to the genus Sporomusa (35). A similarity value of 97.2% was obtained when the 16s rDNA sequences of strain DG-1 and Sporomusa paucivorans DSM 3697² were compared. A similarity value of 95.9% was obtained when the 16s rDNA sequences of strain DG-1 and Sporomusa termidita JSN-2 were compared.
Sporomusa silvacetica sp. nov.

ACKNOWLEDGMENTS

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29. Hermann, M., M. R. Popoff, and M. Sebald. 1987. Sporomusa paucivorans sp. nov., a methylothrophic bacterium that forms acetic acid from hydrogen...


