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

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Sporomusa silvacetica sp. nov. DG-1T (= DSMZ 10669T) (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 ferulate, 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, which was produced in stoichiometries indicative of an acetyl-coenzyme A pathway-dependent metabolism. Fumarate was dissimilated 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 hydrogenase and hydrogenase activities were approximately 9 and 102 µmol min⁻¹ mg of protein⁻¹, respectively. A type II cytochrome was detected in the membrane. The G+C content was approximately 43 mol%. Phylogenetic analysis of the 16S ribosomal DNA indicated that DG-1T 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 dominate 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 CO₂ 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 soil had a pH of approximately 6, a dry weight of 60%, and an 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 ferulate, vanillate, fructose, betaine, fumarate, 2,3-butanediol, pyruvate, lactate, glycerol, ethano- 

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 Geiserberget 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 SL10 (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 Geiserberg, Germany in distilled water (750 ml) for 1 week at 20°C. The liquid was then decanted, centrifuged (10,000 × g, 20 min), filtered 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 deionized, 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), filtered 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 assess N₂-fixing microorganisms (52): a trace element solution (1) (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).

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
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 × g for 20 min at 4°C. The supernatant fluid was transferred to a serum vial (100% N₂ gas phase) and assayed immediately. Hydrogenase and carbon monoxide dehydrogenase activities were assayed at 30°C with Tris-hydrochloride (100 mM, pH 8.5)-benzyl viologen (1 mM)-dithiothreitol (1 mM) in the presence of H₂ (100%) or CO (100%) (19). Polyacrylamide gel electrophoresis (19) was performed in a temperature-controlled Mecaplex anaerobic chamber (100% N₂ gas phase) at 10°C, and in situ staining of gels (19) for carbon monoxide.

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.
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 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 1. Substrate-product stoichiometries for strain DG-1T grown in medium A at 25°C

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Maximum optical density at 660 nm</th>
<th>Amt of substrate consumed (mM)</th>
<th>Amt of product (mM)</th>
<th>Acetateb</th>
<th>Protopate; acetate</th>
<th>Acetate/substrate ratioa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>0.46</td>
<td>4.8</td>
<td>13.8</td>
<td>NA</td>
<td></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>
<td></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>
<td></td>
</tr>
</tbody>
</table>

a Values were corrected for the amount of acetate formed in controls lacking additional substrates.
b The values in parentheses are the theoretical ratios expected for acetogenic consumption of substrates (20).

NA, not applicable.
isolated colony three times. Cultures of DG-lT were considered to be pure based on uniform colony and cellular morphologies.

**RESULTS**

**Enrichment cultures.** Aggregated soil was added to defined (medium B or C), soil extract, and leaf leachate media supplemented with H$_2$-CO$_2$ or vanillate-CO. H$_2$ and CO$_2$ were utilized, vanillate was O-demethylated, and acetate was produced by all soil suspensions within 3 or 4 weeks; repeated transfers yielded stable acetate-producing enrichment cultures. These cultures indicated enrichment of acetogenic consortia occurred. In the leaf leachate and soil extract media, exogenous substrate utilization was retarded; however, acetate production occurred at rates similar to those observed in other media, suggesting that endogenous carbon sources were also utilized.

**Isolation.** Isolate DG-1$^T$ was obtained from a stable medium B enrichment culture containing H$_2$-CO$_2$. DG-1$^T$ produced acetate as the sole soluble end product from utilization of H$_2$. The purity of isolate DG-1$^T$ was ensured by restreaking an isolated colony three times. Cultures of DG-1$^T$ were considered to be pure based on uniform colony and cellular morphologies. Isolate DG-1$^T$ 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-1$^T$ 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-1$^T$ 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-1$^T$ produced acetate concomitantly with growth on fructose, vanillate, or H$_2$-CO$_2$ (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 H$_2$-CO$_2$. In all cases, the substrate was converted to acetate. Protocatechuate and hydroferulate were additional products observed with vanillate and ferulate, respectively. H$_2$ and the O-methyl group of vanillate were utilized as cosubstrates. Fumarate was dismutated to succinate and acetate. Fumarate is also dismutated by *Clostridium acetoclasticum* and *Clostridium formicoacetoclasticum* (18, 41). H$_2$ was not produced. Traces of methane were produced in addition to acetate in cultures grown with H$_2$-CO$_2$; production of trace levels of methane has also been observed with the acetogens *Clostridium thermoautotrophicum* (55), *Acetobacterium woodii* (10), and *Acetobacterium carbinolicum* (10).

**Growth or substrate utilization was observed with cellobiose, glucose, citrate, 4-hydroxybenzaldehyde, xylose, succinate, oxalate, acetate, or CO. According to the results of an API analysis (BioMérieux Deutschland GmbH, Nürtingen, Germany), mannitol, lactose, saccharose, maltose, salicin, arabinose, mannose, melezitose, raffinose, sorbitol, rhamnose, and trehalose were not utilized. Additional results of the API analysis indicated that esculin was hydrolyzed, gelatin was not hydrolyzed, and tryptophanase, urease, catalase, and oxidase activities were absent. Yeast extract (1 g liter$^{-1}$) stimulated growth, but was not required. The growth rates and final cell yields were not appreciably affected when cells were cultivated
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 CO₂, is the preferred terminal electron acceptor for Clostridium thermoaceticum and C. thermoautotrophicum 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-1² 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-1⁷. Sulfate was also not used as an alternative electron acceptor. Strain DG-1⁷ did not fix N₂.

Enzyme activities and membrane spectrum. The carbon monoxide dehydrogenase and hydrogenase activities in cell extracts were approximately 9 and 102 μmol min⁻¹ mg of protein⁻¹, respectively. Two electrophoretically distinct carbon monoxide dehydrogenase bands were observed in situ-stained polyacrylamide gels. A type b cytochrome was detected in membranes; absorption maxima were observed at 432 and 564 nm (Fig. 3A). The cytoplasmic fraction had absorption maxima at 431 and 561 nm (Fig. 3B), indicating that the cytoplasm also contained a type b cytochrome or that a portion of the membranous type b cytochrome was lost to the cytoplasmic fraction during membrane preparation. The chromophoric origin of the absorption maximum at 519 nm in the cytoplasmic fraction (Fig. 3B) was not resolved, but this absorption maximum was characteristic of the β-absorption peak of a type c cytochrome.

GC content and phylogenetic analysis of 16S rDNA. The DNA base composition of DG-1⁷ was 42.7 ± 0.4 mol% G+C (n = 6). Phylogenetic analysis of the 16S rDNA indicated that strain DG-1⁷ was most closely related to the genus Sporomusa (Fig. 4). A similarity value of 97.2% was obtained when the 16S rDNA sequences of strain DG-1⁷ and Sporomusa paucivorans DSM 3697⁷ were compared. A similarity value of 95.9% was obtained when the 16S rDNA sequences of strain DG-1⁷ and Sporomusa termitida JSN-2 were compared.

DISCUSSION

Its cell shape, Gram reaction, formation of spores, and lack of sulfate reduction indicated that strain DG-1⁷ classically belongs to the genus Clostridium. However, thin sections revealed a multilayer cell wall characteristic of gram-negative bacteria. Phylogenetic analysis of the 16S rDNA indicated that DG-1⁷ is most closely related to the genus Sporomusa (Fig. 4). As outlined below, the characteristics of DG-1⁷ are not consistent with the characteristics of previously described acetogenic strains of the genus Clostridium or the genus Sporomusa, and we propose that strain DG-1⁷ is a member of a new acetogenic species, S. silvaecetica; strain DG-1 is the type strain of this new species.

The production of acetate as the primary reduced end product, the stoichiometries of acetate formation observed with fructose, vanillate, and H₂ (approximately 3:1, 3:4, and 1:4, respectively), and carbon monoxide dehydrogenase activity indicated that strain DG-1⁷ is an acetogen. Strain DG-1⁷ differs from other acetogenic clostridia (12, 20, 56) as follows: it differs from C. aceticum, Clostridium fervidus, C. formicoaceticum, Clostridium magnus, and Clostridium sp. strain CV-AA1 in its Gram reaction; it differs from C. fervidus, C. formicoaceticum, Clostridium ljungdahlii, Clostridium mayomega, Clostridium (Oxobacter [15]) pfennigii, C. thermoaceticum, and C. thermoautotrophicum in its optimum temperature and temperature range; it differs from C. magnum in size; it differs from all other acetogenic clostridia except Sporomusa species in cell shape during spore formation; it differs from all other acetogenic clostridia in its substrate range (i.e., it differs in specific substrates utilized on a case-by-case basis); it differs from C. formicoaceticum in N₂ fixation (5); and it differs from all other acetogenic clostridia in its G+C content.

The cell and spore morphologies of strain DG-1⁷ were typical of the genus Sporomusa (45). The following six Sporomusa species have been characterized previously: Sporomusa acidovorans (47), Sporomusa malonica (17), Sporomusa ovata (45), S. paucivorans (29), Sporomusa sphaleroides (45), and S. termitida (8). To date, only the 16S rDNAs of S. paucivorans and S. termitida have been sequenced. In contrast to strain DG-1⁷, S. paucivorans does not utilize sugars, requires yeast extract for growth, has a G+C content of 47 mol%, and has not been observed to form spores (29). In contrast to DG-1⁷, S. termitida does not utilize fructose, fumarate, or glycerol and has a G+C content of 49 mol%. On a case-by-case basis, DG-1⁷ differs from the other Sporomusa species in spore morphology, substrate range, yeast extract requirement, and G+C content. A type b cytochrome was detected in the membranes of DG-1⁷, a trait characteristic of other Sporomusa species (8, 17, 45). Betaine stimulates the production of membranous type b cytochromes in S. ovata (33).

Strain DG-1⁷ is the first Sporomusa strain to be isolated from well-drained terrestrial soils, a habitat that is subject to fluctuations in aeration and redox potential. With the exception of S. termitida, all other Sporomusa species have been isolated from habitats that are classically considered to be anaerobic (e.g., freshwater sediments) (20). S. termitida was isolated from a termite gut (8), a microhabitat also subject to fluctuations in redox potential and anaerobiosis (9). It thus appears that certain Sporomusa species can adapt to habitats that are not strictly anaerobic. The origin of DG-1⁷ is also in marked contrast to the origins of numerous other mesophilic acetogens isolated from sewage, gastrointestinal tracts of animals, and water-logged habitats, such as marine sediment and ditch mud (20, 56).

Acetate is a major organic acid of mineral forest soils (59). Although acetate does not accumulate in situ, forest soils produce acetate and CO₂ as major carbonaceous products during the turnover of endogenous organic matter in anaerobic microcosms (35). The stoichiometry of acetate production from exogenously added H₂ by anaerobically incubated mineral soils (35, 63) suggests that acetate can be formed via the acetyl-CoA Wood-Ljungdahl pathway (20). The isolation of an acetogen from soil in Geisberg, Germany, confirms that acetogens are present and potentially active in anaerobic microsites in aggregated forest soils.

The degradation of lignocellulose yields lignin monomers, such as vanillate, syringate, and ferulate (14, 25). Strain DG-1⁷ exhibited a broad substrate range and utilized methoxylated lignin derivatives in addition to sugars, alcohols, organic acids, betaine, and H₂-CO₂. In contrast to C. aceticum and C. formicoaceticum (27), strain DG-1⁷ did not grow at the expense of 4-hydroxybenzaldehyde. A number of acetogens can utilize multiple substrates simultaneously (7, 38, 41). In addition, most acetogens can utilize electron acceptors other than CO₂ for the conservation of energy (20, 21, 56). Strain DG-1⁷ used H₂ and the methoxyl groups of vanillate as cosubstrates, a metabolic capacity that may enhance the in situ activities of certain acetogens (38). Strain DG-1⁷ did not engage in lactate fermentation concomitant with acetogenesis, as Peptostrepto-
**SPOROMUSA SILVACEATICA SP. NOV.**

Sporomusa silvaceatica sp. nov., a methylotrophic bacterium that forms acetic acid from hydrogen, was isolated from a horizon of a beech forest soil in Bavaria, Germany, and has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as strain DSMZ 10669.

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