Meniscus, a New Genus of Aerotolerant, Gas-Vacuolated Bacteria

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Two gram-negative, gas-vacuolated, aerotolerant, heterotrophic bacteria were isolated from the anaerobic digester of the Göttingen Waste Treatment Plant, Göttingen, Federal Republic of Germany. Pure white colonies of these organisms were picked from pour plates of basal growth medium containing 1.2% agar. The plates had been inoculated with digester sludge and incubated in a nitrogen-carbon dioxide atmosphere using the agar itself as the main carbon and energy source. One organism was rod shaped, and the other was vibrioid; both were encapsulated and nonmotile. The two organisms have identical deoxy-ribonucleic acid base compositions and identical nutritional requirements and fermentation characteristics. It is proposed that these organisms are stable morphovariants of a single species belonging to a new genus, for which the name *Meniscus* is proposed. The name proposed for the species (the type species) is *Meniscus glaucopsis* sp. nov., of which the type strain is strain V (= ATCC 29398). The genus *Meniscus* does not appear to belong to any of the currently recognized families of bacteria.

Gas vesicles are found primarily in procaryotic organisms from aquatic habitats. They are particularly common in phototrophic organisms such as the blue-green bacteria and the green and purple sulfur bacteria (4), but they have also been reported in a number of heterotrophic bacteria (9-11). Although bacteria containing gas vesicles have been demonstrated in the hypolimnetic layer of some lakes (2, 5, 12), successful cultivation of these hypolimnetic heterotrophs in the laboratory has not been reported in the literature.

The colonies of the organisms reported here were first observed on agar pour plates designed for the counting of phototrophs. The medium had been inoculated with sludge from the anaerobic digester of the Göttingen Waste Treatment Plant, Göttingen, Federal Republic of Germany. The organisms were recognized as being unusual, and it was decided to attempt their isolation and classification.

MATERIALS AND METHODS

Bacterial strains. The organisms were isolated from anaerobic digester sludge collected in sterile jars at the Göttingen Waste Treatment Plant, Göttingen, Federal Republic of Germany. The samples were diluted 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} with sterile distilled water. One milliliter of each dilution was added to 100 ml of maltose-free basal growth medium (BGM) plus 1.2% agar kept at 40°C, and the agar was poured into four petri dishes. After 2 weeks of anaerobic incubation at 30°C, chalky white colonies were observed on the plates. The agar under

and surrounding the colonies was soft due to hydrolysis of the agar. The plates prepared from the 10^{-1} dilution contained about 200 colonies each. Phase-microscopy examination revealed that some colonies consisted of rods whereas others consisted of vibrios. Most of the cells contained bright, refractile regions thought to be gas vacuoles. Each colony type was purified by streaking on BGM agar plus 0.5% maltose. The strains were designated "R" for rod and "V" for vibrio.

Cultures were maintained at 4°C in BGM deeps containing 0.3% agar, 0.2% maltose, and 1.0% CaCO_3 . The tubes were rendered anaerobic by means of cotton plugs containing 0.5 ml of concentrated Na_2CO_3 and 0.5 ml of 20% pyrogallol acid. They were then sealed with rubber stoppers (8). The two organisms were run in parallel in all experiments. The vibrioid strain was deposited with the American Type Culture Collection (ATCC), Rockville, Md., under the number 29398.

Media. BGM contained (per liter): yeast extract, 1.0 g; KH_2PO_4 , 0.5 g; NaCl , 0.4 g; NH_4Cl , 0.4 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 g; sodium thioglycolate, 0.3 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 g; trace elements solution (TES), 1.0 ml. The TES, modified from Pfennig's formula (personal communication), contained (per liter): $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.10 g; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.03 g; H_3BO_3 , 0.3 g; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2 g; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 g; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.02 g; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.03 g; pH 3 to 4.

The pH of the BGM was adjusted to 7.3 with 10% Na_2CO_3 before autoclaving, or the medium was autoclaved with the pH unadjusted and then adjusted to about pH 7.0 after autoclaving by the addition of 2.0 ml of filter-sterilized 5.0% NaHCO_3 per 100 ml of BGM. All solid media contained 1.0% CaCO_3 and 1.5% agar. Stock medium contained 0.3% agar. Mal-

tose, at a concentration of 0.2 to 0.5%, was, in general, used as the carbon source. Large volumes of liquid cultures were sparged with N_2 gas for 15 min prior to and after inoculation. Smaller volumes in test tubes were rendered anaerobic by means of 20% pyrogallic acid and concentrated Na_2CO_3 (8) or by the use of Hungate tubes sparged with N_2 gas prior to autoclaving. Resazurin, at a concentration of 0.1 mg/100 ml, was used as an oxidation-reduction indicator. Agar plate cultures were grown in anaerobic jars (BBL) or in glass jars filled with 20% CO_2 and 80% H_2 or N_2 gas.

Techniques of microscopic observation. Phase photomicrographs were taken on 35-mm high-contrast copy film (Kodak) with a Cannon FTB camera attached to a Zeiss RA38 microscope. The presence of gas vacuoles was demonstrated by the disappearance of the vacuoles upon the application of a sharp blow with a hammer to a wet mount of the cells. The cover slip was protected with a 3- to 4-mm-thick rubber pad. Phase-microscopic examination of wet mounts made with dilute India ink was used to demonstrate capsules.

A Hitachi HU-11E electron microscope was used for observation of sectioned cells. Concentrated suspensions of bacteria from agar plates were fixed at 4°C for 30 min in large volumes of phosphate-buffered (pH 6.8) glutaraldehyde (2.5%). These suspensions were mixed with equal volumes of warm 4% agar and then cut into 1-mm cubes. The agar-embedded bacteria were postfixed at 4°C in phosphate-buffered (pH 6.8) OsO_4 (1%) for 1 h. The blocks were dehydrated in alcohol and embedded in Araldite 502. Thin sections were stained with uranyl acetate and lead citrate.

Methods of bacteriological characterization. Unless otherwise specified, all inocula used in specified tests were taken from young cultures grown on BGM agar plates plus 0.5% maltose and suspended in BGM without yeast extract. All test cultures were incubated for 7 to 30 days at 30°C and examined with a microscope for contamination before a particular test was confirmed. All tests were run in duplicate. BGM plus 0.2% maltose and without yeast extract was used for the determination of vitamin requirements. All glassware for this determination was acid cleaned. Forty-five carbon sources were tested for utilization at a concentration of 0.2% (wt/vol). Growth at various temperatures was determined by using BGM agar plus 0.5% maltose. Incubation at 5, 10, 15, 20, 25, 30, 35, and 40°C was extended for 30 days.

The initial pH for growth was determined by adjusting the pH of BGM agar plus 0.5% maltose with dilute HCl or NaOH prior to inoculation. This value was recorded as the growth pH. Aerobic growth was tested on BGM agar plates plus 0.5% maltose. Macromolecular hydrolysis was tested on BGM agar containing 0.5% maltose plus the proper ingredient or in test tubes with the agar and the maltose left out. The following were tested on plates: starch, 0.5%; tributyrin, 1.0%; cellulose, 0.3%; xylan, 0.3%; chitin (6), 1.0%; deoxyribonucleic acid (DNA), 0.2%; and casein, 0.5%. Hydrolysis of these compounds was indicated by the formation of a clear zone

around the colony. Starch and DNA hydrolyses were demonstrated with the aid of Lugol iodine and 1-N HCl, respectively. Tested in tubes were the following: glycogen, 0.5%; inulin, 0.2%; pectin, 0.3%; gum arabic, 0.2%; and dextrin, 0.2%. Since BGM alone does not support growth of these organisms, hydrolysis was indicated by growth (turbidity) and acid production (drop in pH). A test for hydrolysis of agar, 0.3%, and gelatin, 5.0%, was done in test tubes. Hydrolysis was demonstrated by the inability of the culture medium to solidify when immersed in ice water after incubation.

Catalase and oxidase were tested for by dropping 3.0% H_2O_2 and 1.0% *p*-amino dimethylaniline oxalate, respectively, onto colonies on BGM agar plus 0.5% maltose. Deaminase and urease were tested for by growing the cells in media containing Casamino Acids and urea, respectively. Production of ammonia was checked with Nessler reagent. The test for acetylmethylcarbinol (AMC) production was performed by adding 40% KOH containing 0.3% creatine and 5% alpha-naphthol (in 95% ethanol) to a maltose-grown culture.

Nitrate reduction was tested in BGM containing 0.2% maltose and 0.5% KNO_3 . The formation of nitrate was checked by the addition of sulfanilic acid (8.0 g in 1,000 ml of 5.0 N acetic acid) and dimethyl-alpha-naphthylamine (5.0 g in 1,000 ml of 5.0 N acetic acid).

Indole production was determined by growing the cells in BGM + 0.05% maltose and 0.5% tryptophan and by adding Kovac reagent (5.0 g of *p*-dimethylaminobenzaldehyde in 75 ml of amyl alcohol and 25 ml of concentrated HCl) to the grown culture. The production of H_2S was tested for in SIM medium (Difco) plus 0.1% yeast extract and vitamin B_{12} .

DNA was extracted by the procedure of Marmur (7), and its mean base composition (moles percent guanine plus cytosine [G+C]) was determined by Mandel using the buoyant density CsCl method (7).

RESULTS

Morphological characteristics. Figures 1 and 2 show phase-contrast photomicrographs of strains "R" and "V," respectively. The rods measured approximately 0.7 by 2.0 μm , and the vibrios were about 0.7 μm wide with an outer diameter of 3.0 μm . Both organisms were encapsulated, gram negative, and nonmotile.

The cells of both strains contained bright, refractile regions of irregular outline. Figure 3 shows an electron micrograph of the rods. The individual vesicles of the gas vacuole were clearly visible.

Cultural characteristics. On agar plates, both strains produced colonies with a circular form, an entire margin, convex elevation, and a smooth, glistening surface. The colonies appeared translucent or opaque, chalky white. As previously found by Van Ert and Staley (10), the variation in colony opacity is apparently due to variation in the number of gas vacuoles

FIG. 1. Phase photomicrograph of strain R, the rod-shaped isolate. Note the refractile appearance of the gas vacuoles. Marker indicates 2 μ m.

FIG. 2. Phase photomicrograph of strain V, the vibrioid isolate. Note the refractile appearance of the gas vacuoles. Marker indicates 2 μ m.

within the individual cells of the colony. The translucent colonies contained fewer cells with gas vacuoles. In agar shakes, the colonies were compound lenticular (Y shaped). Cells of both organisms grew evenly suspended in anaerobic liquid media on a magnetic stirring base. In stationary cultures, the cells remained fairly evenly suspended or collected at the surface. Refrigeration especially enhanced the tendency of the cells to collect at the surface. This was mainly due to the increased density of water at 4°C. At no time was the formation of pellicles observed.

Growth characteristics. Both strains grew in the 15 to 35°C range. They did not grow at 10 and 40°C. The optimum pH for growth of both organisms was between 7.0 and 8.0. There was

little growth of either organism below pH 6.0 or above pH 10.0.

Both strains grew under aerobic conditions on BGM agar plus 0.5% maltose provided the concentration of CO₂ was at least 1%. They also grew in a candle jar. It was noted that the colonies of cultures grown under aerobic conditions stuck to the agar and had a rubbery consistency, whereas the cultures grown under anaerobic conditions had a buttery consistency. Both strains tested negative for catalase, oxidase, deaminase, urease, acetylmethylcarbinol, indole, H₂S, and nitrate reduction.

Nutrition. Both organisms grew well on BGM plus maltose. Thiamin, cobalamine (vitamin B₁₂), and CO₂ were required for growth. There also appeared to be a need for ammonium ion or yeast extract. Both organisms grew in BGM plus maltose when yeast extract was replaced with a vitamin mixture (B vitamins plus biotin and nicotinic acid) or when NH₄Cl was replaced with vitamin-free Casamino Acids. However, neither organism grew when both the yeast extract and the NH₄Cl were replaced with vitamin mixture and Casamino Acids. These results can be explained by the inability of either strain to carry out deamination, ammonia being required for the synthesis of purines and pyrimidines. The latter compounds are provided in media by the yeast extract.

Both organisms showed identical responses to 45 carbon sources tested for the ability to serve as carbon and energy sources for growth. Both organisms fermented melezitose, raffinose, cellobiose, sucrose, lactose, maltose, melibiose, trehalose, fructose, galactose, glucose, rhamnose, CH₃- α -D-glucoside, esculin, salicin, D-ribose, D-xylose, and D-arabinose. They did not ferment mannose, sorbose, glycerol, mannitol, sorbitol, adonitol, dulcitol, inositol, or amino acids.

Neither organism could hydrolyze starch, cellulose, DNA, gelatin, casein, pectin, inulin, gum arabic, tributyrin, chitin, xylan, or glycogen. Both organisms were able to hydrolyze agar and dextrin. End products formed from the fermentation of glucose were acetic, propionic, and succinic acids.

The G+C content of the DNA for both organisms was 44.9 mol%.

DISCUSSION

Nutrition. Strains "R" and "V" responded identically to the organic substrates tested. Both had a fermentative metabolism and utilized the same substrates. Large amounts of acid but no gas were formed. Their vitamin re-

FIG. 3. Electron micrograph of a longitudinal section of the rod-shaped cell resolving the fine structure of the vesicles comprising the gas vacuoles. The diameter of the vesicles measures approximately 100 nm. Marker indicates 1.0 μ m.

quirements and their DNA base compositions also were identical.

Morphological characteristics. Both strains were nonmotile, encapsulated, and aerotolerant. Strain "R" contained rod-shaped cells which measured 0.7 by 2.0 μ m. The cells occurred mostly singly, but some pairs and chains of cells could be observed. Strain "V" contained vibrioid cells about 0.7 to 1.0 μ m thick and often gave the appearance of forming rings due to the overlapping ends. The outer "ring" diameter was about 3.0 μ m. Under certain undefined conditions, long, tightly coiled spirals, S shapes, and pretzel shapes could be observed.

For the purpose of classification, it would be of interest to compare the two new isolates to other heterotrophs that form gas vacuoles. One may mention the genera *Halobacterium* (1), *Anacalomicrobium* (1, 9), *Prosthecomicrobium* (1, 9), and *Microcycilus* (1, 3, 10), the members of which have been cultivated, and *Pelonema* (1), *Peloploca* (1), *Pelosigma* (1), and *Brachyarcus* (1), the members of which have not been cultivated.

The two new isolates do not fit the description of the first four genera mentioned above for the following reasons: both species of the genus *Halobacterium* are obligately aerobic halophiles with a G+C content of the major DNA component of 66 to 68 mol%. *Anacalomicrobium* and *Prosthecomicrobium* are both prosthecate bacteria. *Anacalomicrobium* contains one species, a facultative anaerobe with a DNA G+C content of 70.4 mol%. The species of the genus

Prosthecomicrobium are strict aerobes and have a DNA G+C content of 65.8 to 69.9 mol%. *Microcycilus* is not a well-defined genus, and the G+C content of the DNA ranges from 39.5 to 68.4 mol%. The G+C contents of the new isolates fit within this range. However, the three species of *Microcycilus* are all catalase positive, strict aerobes, whereas the new isolates are catalase-negative, aerotolerant anaerobes with a CO₂ requirement.

Since members of *Pelonema*, *Peloploca*, *Pelosigma*, and *Brachyarcus* have not been isolated and cultivated in pure culture, a comparison of the new isolates with these organisms must of necessity be limited to morphological characteristics. *Pelonema* and *Peloploca* species all form long multicellular filaments, up to 600 μ m long, and propagation is by fragmentation of the filaments. These characteristics have not been observed in the new isolates. The two *Pelosigma* species have been observed to form sigmoid aggregates of four cells and multiples of four cells. This characteristic also has not been observed in the new isolates. The vibrioid isolate has many characteristics in common with the genus *Brachyarcus*, first described by Skuja (1) in 1964. Both organisms are bent like a bow, are colorless with gas vacuoles, and have encapsulated cells. However, whereas *Brachyarcus thiophilus*, the type species of *Brachyarcus*, has occasionally been observed to accumulate minute sulfur granules (1), the new isolates have not been observed to form sulfur granules, even when grown with small

amounts of H_2S in the atmosphere.

It is evident from the discussion above that the mere presence of gas vacuoles in cells cannot serve as a unifying taxonomic characteristic. One must, therefore, consider the possible relationship of the new isolates to nonvacuolated vibrioid organisms, the most likely families being the *Spirillaceae* and the *Vibrionaceae*. As the members of *Spirillaceae* are oxidase positive and incapable of fermenting carbohydrates, the new isolates must be excluded from this family. Although members of the family *Vibrionaceae* are facultative anaerobes, they are also oxidase and catalase positive, and they carry out respiration in the presence of oxygen. These characteristics eliminate the new isolates from membership in this family. The new isolates do not carry out respiration in the presence of oxygen, as indicated by the same amount of growth being obtained under both aerobic and anaerobic conditions. Because the difference in morphology is the only apparent difference between the new isolates, it is proposed that these organisms are merely morphovars (morphotypes) of the same species. It is further proposed that this organism be placed in a new genus, *Meniscus* (Gr. n. *meniskos* crescent moon), and that the species be named *M. glaucopsis* sp. nov. (Gr. adj. *glaucopsis* gleaming eyed).

Meniscus gen. nov. Curved or straight rods, 0.7 to 1.0 by 2.0 to 3.0 μm . Nonmotile. Resting stages not known. Encapsulated. Gas vacuoles arranged at random within the cells. Gram negative. Colonies chalky white. Chemoorganotrophic; fermentative metabolism with no gas production. Facultatively anaerobic with a CO_2 requirement. Vitamins B_{12} and thiamin are required for growth. Temperature for optimum growth: 30°C; no growth at 10 or at 40°C. The G+C content of the DNA is 44.9 mol%. The type species is *M. glaucopsis* sp. nov.

Meniscus glaucopsis sp. nov. Unicellular, gram negative, vibrioid or rod-shaped cells, 0.7 to 1.0 by 2.0 to 3.0 μm , colorless with gas vacuoles. Cultures may show single cells, pairs, tightly coiled spirals, S shapes (two cells, one inverted), and doughnut-shaped cells, where the ends are overlapping before division by binary fission has occurred. The cells are encapsulated. No resting stages known.

Colonies are circular, convex in elevation, with an entire margin and smooth, glistening surface. The colonies may appear translucent or opaque, chalky white. The larger the number of gas vacuoles within the cells of the colony, the whiter the colony. The consistency of the colonies is buttery when grown anaerobically and rubbery when grown aerobically.

Aerotolerant anaerobic with a CO_2 requirement. Catalase and oxidase negative. Does not carry out respiration. Nitrate not reduced. Optimum growth occurs around 30°C at pH 7.0. Growth occurs at 15 and 35°C, but not at 10 or 40°C. Cobalamine (vitamin B_{12}), thiamin, and CO_2 are required for growth. Good growth in defined medium occurs with ammonium as the nitrogen source.

The following characteristics were negative: deaminase, urease, acetylmethylcarbinol, indole, and H_2S production and nitrate reduction.

Ferments agar, dextrin, melzitose, raffinose, cellobiose, sucrose, lactose, maltose, melibiose, trehalose, fructose, galactose, glucose, rhamnose (weakly), CH_3 - α -D-glucoside, esculin, salicin, D-ribose, D-xylose, and arabinose.

Does not ferment mannose, sorbose, glycerol, lactate, mannitol, sorbitol, adonitol, dulcitol, inositol, or amino acids.

Does not hydrolyze starch, cellulose, DNA, gelatin, casein, pectin, inulin, gum arabic, tributyrin, chitin, xylan, or glycogen.

Fermentation end products are acetic, propionic, and succinic acids.

The G+C content of the DNA is 44.9 mol%.

Isolated from anaerobic digester sludge, but probably also present in anaerobic hypolimnion of lakes.

Type strain: strain V (= ATCC 29398).

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REPRINT REQUESTS

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