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Hydrophobicities and Electrophoretic Mobilities of Anaerobic Bacterial Isolates from Methanogenic Granular Sludge

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The hydrophobicities and electrophoretic mobilities of isolates from methanogenic anaerobic granular sludge were measured and compared with those of strains from culture collections. All new isolates were highly hydrophobic, indicating that the upflow anaerobic sludge blanket reactor concept selects for hydrophobic bacteria. *Methanothrix soehngenii*, a methanogen often observed in methanogenic granular sludge, was highly hydrophobic and showed low electrophoretic mobility at pH 7. The role of this strain in the formation of methanogenic granular sludge is discussed.

Granular methanogenic sludge consists of a densely packed structure of anaerobic bacteria. Such microbial aggregates are preferentially formed in upflow anaerobic sludge blanket (UASB) reactors (8). The adhesion properties of bacteria present in high numbers in granular sludge adapted to ethanol or propionate were measured and compared with those of culture collection strains. Adhesion properties of cells play an important role in initial immobilization. Adhesion is strongly dependent on the surface characteristics of bacteria. Surface hydrophobicity and electrophoretic mobility may be taken as an indicator for the adhesion properties of bacteria (15, 16).

Methanobrevibacter arboriphilicus (DSM 744), *Methanospirillum hungatei* (DSM 864), *Methanosarcina barkeri* (DSM 800), *Pelobacter carbinolicus* (DSM 2909), *P. carbinolicus* (DSM 2984), and *Pelobacter propionicus* (DSM 2379) were obtained from the Deutsche Sammlung von Mikroorganismen (DSM), Braunschweig, Germany. *Methanothrix soehngenii* was the Opfikon strain isolated by Huser et al. (5). Granular sludge originating from a 30-m^3 UASB reactor at the CSM sugar refinery in Breda, The Netherlands (10) was cultivated with ethanol or propionate used as substrates under well-defined conditions (2).

Isolation of bacteria was started by disintegration of methanogenic granules with a syringe without a needle, followed by homogenization with a glass Potter homogenizer (Tamson, Zoetermeer, The Netherlands). Serial dilutions were made in triplicate in growth medium. Bottles were incubated at 37°C for 3 months with ethanol, H_2-CO_2, or formate as the sole carbon and energy source. The highest dilution in which growth was observed was diluted in 2% agar medium by using the roll tube technique as described by Hungate (4). Pure cultures were obtained by repeating the roll tube procedure. Finally, single colonies were picked and transferred to liquid medium. Purity was checked by microscopy, colony morphology, and growth in complex medium containing yeast extract and glucose. Strains were routinely cultivated in 120-ml vials containing 50 ml of bicarbonate-buffered medium and a gas phase of N_2-CO_2 (4:1) if acetate, ethanol, or formate (20 mM) was the substrate or H_2-CO_2 (4:1) if H_2-CO_2 was the substrate. Both atmospheres were at 1.8 atm (ca. 182 kPa). Strain EE121 was isolated from methanogenic granules originating from an UASB-reactor that was fed ethanol as the sole carbon and energy source (11). Bacteria of this strain were gram positive and rod shaped with pointed ends, and they formed spores. They were between 3.5 and 10 μm in length and about 1 μm in width. Cells moved by lateral flagellation. Antiserum raised against EE121 did not react with other isolated strains (PHC and EF strains). The following hydrogenotrophic methanogenic strains were isolated from propionate-grown granular sludge with H_2-CO_2 as a substrate: PHC 252, PHC 254, PHC 256-1, PHC 256-2, PHC 259-1, PHC 259-3, and PHC 2511. These strains produced methane from H_2-CO_2; were gram negative, nonmotile, and non-spore forming; and showed autofluorescence at 420 nm. Cells were rod shaped, 0.5 to 1 μm wide and 4 to 10 μm long. Antisera were prepared against strain PHC 252 and *M. arboriphilicus* AZ (DSM 744). Both antisera showed clear cross-reactions with *M. arboriphilicus* AZ (DSM 744) and all PHC strains, indicating that the isolated strains belong to the genus *Methanobrevibacter*. From ethanol-grown granular sludge the isolates EF 173, EF 174, and EF 175 were obtained, with formate as the substrate. These strains produced acetate from formate; were gram positive, nonmotile, and spore forming; and showed no autofluorescence at 420 nm. Cells were rod shaped, 1.5 μm wide and 6 to 9 μm long. Spores were 1.3 μm in width and 2 to 3 μm in length.

Bacterial hydrophobicity was quantified by the contact angle measurement method as described by van Loosdrecht et al. (15). Electrophoretic mobility was measured by laser Doppler velocimetry with a ZetaSizer (Malvern Instruments, Malvern, England) at pH 7 and pH 2 in 0.01 M phosphate-buffered saline (0.029 g of KH_2PO_4, 0.119 g of K_2PO_4, and 0.493 g of NaCl per liter). In this procedure, the migration velocity of bacteria in an electric field in an electrophoresis cell is determined by measuring the Doppler effect from a laser beam (16).

The hydrophobicity and electrophoretic mobility at two pH values were measured as indicators for the adhesion properties of the isolates. The obtained values were compared with those of some culture collection strains (Table 1). All isolates from granular sludge had relatively high contact angles and thus were rather hydrophobic, especially if their contact angles were compared with those of aerobic culture collection strains. For 20 aerobic strains, Loosdrecht et al. (15) found contact angles of between 20.1 and 44.7°. The

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TABLE 1. Contact angles and electrophoretic mobilities of several isolates from granular methanogenic sludge and of anaerobic bacteria

Bacterial strain or species	Contact angle[a] (°)	Electrophoretic mobility[b] (10^{-8} m · V^{-1} · s^{-1})	
		pH 7	pH 2
PHC 254	73.6	−2.14	−0.26
PHC 256-1	75.5	−1.65	−0.07
PHC 256-2	76.5	−2.30	−0.17
PHC 259-1	73.8	−2.33	−0.28
PHC 259-3	73.0	−2.04	0.11
PHC 2511	72.5	−2.12	1.01
EF 173	69.3	−2.51	0.32
EF 174	68.4	−1.92	0.51
EF 175	78.5	ND[c]	ND
EE121	82.0	−2.30	0.77
M. arboriphilicus AZ	53.8	−1.18	−0.19
M. hungatei JF1	31.8	−2.19	0.29
M. soehngenii	69.4	−0.32	0.60
M. barkeri	71.4	−2.40	ND
P. carbinolicus RE1	70.0	−2.88	0.81
P. carbinolicus BE4	86.1	−3.71	1.80
P. propionicus	49.0	−3.12	1.12

[a] Mean of six measurements; average standard deviation, 1.4°.
[b] Average standard deviation, 0.15 × 10^{-8} m · V^{-1} · s^{-1}.
[c] ND, not determined.

PHC strains, which cross-reacted with the antiserum of *M. arboriphilicus* AZ, were more hydrophobic than *M. arboriphilicus* AZ, which is a culture collection strain. Continuous growth of strain AZ in suspension and repeated transfers for many years before deposition at the German type culture collection, the DSM (19), may have selected for a less hydrophobic variety. Classical enrichment (batch and chemostat) and isolation techniques select for bacteria that detach easily from particles (soil, sediment, particular matter in water, etc.), grow as homogeneous suspensions, and form single colonies, preferably from single cells, on solid media. It is therefore not surprising that hydrophilic or highly charged hydrophobic bacteria are generally isolated by classical methods. Indeed, a lower contact angle was found for *P. propionicus* isolated by these classical techniques from freshwater sediment (13) than was found for the newly isolated anaerobic strains. In granular sludge, a selection for well-adhering (hydrophobic) bacteria has already taken place. The chance to isolate hydrophobic bacteria was enhanced in this study by directly isolating the most numerous bacteria from granular sludge after mechanically breaking up the granules. By following this strategy, a counterselection for homogeneously growing, nonadhering bacteria could be prevented. The high hydrophobicity of the two *P. carbinolicus* strains isolated from granular sludge (1) is another indication of the selection of well-adhering bacteria by this type of sludge.

The electrophoretic mobility of each of the anaerobic strains is different (Table 1). In wastewater with a relatively high ionic strength, the charge of the bacteria is of less importance. Charge becomes a determining factor for adhesion only at ionic strength below 10 mM (17). *M. soehngenii*, an acetoclastic methanogen, is often observed in large numbers in methanogenic granular sludge and methanogenic biofilms (1, 3, 7, 9, 12, 14). The other important acetoclastic methanogenic bacterium, *M. barkeri*, has a much lower affinity for acetate than *M. soehngenii* (6) and is present at much lower numbers in methanogenic granular sludge of UASB reactors run for a long period at low-acetate effluent concentrations (3). *M. soehngenii* is very hydrophobic and almost uncharged at pH 7 (Table 1). It also forms long intertwining threads in which other bacteria or inorganic precipitates are entangled. These growth kinetic properties and physicochemical properties, as well as its shape, make *M. soehngenii* the ideal organism for adherence and give *Methanothrix* spp. a key role in stabilizing microbial agglomerates like granular sludge or biofilms (3, 18).

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