A Six-well Plate Method: Less Laborious and Effective Method for Cultivation of Obligate Anaerobic Microorganisms

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We developed a simple, less laborious method to cultivate and isolate obligate anaerobic microorganisms using a six-well plate together with the AnaeroPack System, designated as the six-well plate method. The cultivation efficiency of this method, based on colony-forming units, colony formation time, and colony size, was evaluated with four authentic obligate anaerobes (two methanogenic archaea and two sulfate-reducing bacteria). The method was found to be comparable to or even better than the roll tube method, a technique that is commonly used at present for the cultivation of obligate anaerobes. Further experiments using 21 representative obligate anaerobes demonstrated that all examined anaerobes (11 methanogens, 5 sulfate- or thiosulfate-reducing bacteria, and 5 syntrophs) could form visible colonies on the six-well plate and that these colonies could be successfully subcultured in fresh liquid media. Using this method, an unidentified sulfate-reducing bacterium was successfully isolated from an environmental sample.

Key words: methanogen, sulfate-reducing bacterium (SRB), gellan gum, uncultured anaerobes

Strictly anaerobic microorganisms are one of the major populations in a wide variety of anoxic ecosystems, such as freshwater and marine sediments, terrestrial and oceanic subsurfaces, and the guts of insects and animals. In fact, a previous study estimated that over 90% of prokaryotic microbes reside in anaerobic environments (23). Recent molecular ecological studies have also demonstrated that anaerobic microorganisms are phylogenetically quite diverse, and that the majority have not been cultured yet; therefore, their genome structures, physiology, and in situ functions are largely unknown (references in a review article by Sekiguchi) (18).

One of the critical reasons why the majority of anaerobes remain uncultured is that anaerobic microorganisms, in particular obligate anaerobic microorganisms, require special apparatus and laborious techniques for their isolation and cultivation. They are mostly acknowledged as being oxygen (and reactive oxygen species) susceptible and require very low redox potential for respiration with a variety of terminal electron acceptors other than oxygen, as is the case for sulfate-reducing bacteria (SRB), methanogenic archaea, and homoacetogens. The removal of oxygen from medium by purging and then replacing with O2-free gases together with the addition of reducing agents to the medium facilitates the growth of obligate anaerobic organisms. Two major methods, namely, roll tube and deep agar methods, are often used for the isolation and cultivation of anaerobes. Both canonical methods are conducted using glass tubes or vials with butyl rubber stoppers and aluminum caps to keep the inner gas phase strictly anaerobic.

Here, we have developed a simple plate cultivation method for obligate anaerobic microorganisms, such as methanogens, SRB, and hydrogen-producing syntrophs, using a commercially available six-well plastic plate and the AnaeroPack system, which are often used for the isolation and/or enumeration of readily cultivable heterotrophic anaerobes (e.g., in 7, 19). We have examined and evaluated the advantages of this method for the isolation and cultivation of obligate anaerobes.

Materials and Methods

Chemicals and gases

All chemicals used in this study were of analytical grade. Mixed gases used for medium preparation were N2/CO2 (80:20, v/v; purity, ≥99.995%) and/or H2/CO2 (80:20, v/v; purity, ≥99.995%), unless otherwise indicated. All gases were deoxidized with a Deoxidized Gas Pressure Injector (IP-8; Sanshin Industrial, Yokohama, Japan) unless otherwise indicated. Deionized and distilled water was used for the preparation of reagents and culture media.

Strains and cultivation conditions

Anaerobic microorganisms used in this study are listed in Table 1. For liquid preculture, basal medium, designated as W medium, which is a slightly modified medium based on that of Widdel and Pfennig, was used (24). Medium was prepared as described in Plugge (16). Na2S·9H2O and cysteine-HCl·H2O, which were degassed and sterilized in an autoclave, were added to medium as a reductant. Both reductants were added at a final concentration of 0.3 g L−1 (except for Desulfotomaculum nigrificans, at 0.12 g L−1) for all
cultivations described below unless otherwise indicated. Substrates and temperatures for the cultivation of each strain are shown in Table 1. A liquid culture of each strain in the mid-exponential or early stationary phase as determined by CH₄ production or optical density was used for the following growth tests of six-well plate and roll tube methods.

**Comparison of cultivation efficiency between six-well plate and roll tube cultivation methods using representative anaerobes**

Cultivation efficiency was compared in terms of colony-forming units (CFU), incubation time for colony development, and colony diameter after appropriate incubation between six-well plate and roll tube cultivations. The six-well plate method was examined using two methods of inoculation: one performed on a clean bench (organisms were inoculated under aerobic conditions, hereafter referred to as aerobic inoculation) and the other in an anaerobic chamber (hereafter referred to as anaerobic inoculation). Two methanogens (thermophile and mesophile) and two sulfate-reducing bacteria (SRB) (thermophile and mesophile) were tested as representatives of obligate anaerobes, namely, *Methanothermobacter thermautotrophicus*, *Methanoculleus bourgensis*, *Desulfotomaculum thermosarovorans*, and *Desulfovibrio vulgaris* subsp. vulgaris strain Hildenborough (hereafter referred to as *D. vulgaris*). Serial dilutions of precultures of these strains were prepared with reduced W medium in an anaerobic chamber (Don Whitley Scientific Limited, West Yorkshire, England) under an N₂/H₂/CO₂ atmosphere (80:10:10, v/v) and were inoculated into the six-well plates and roll tubes according to the procedures described below. Examinations were performed in triplicate. CFUs of the tested strains were determined from the average number of colonies that appeared in the range of 30–160 on the six-well plate wells or of 30–2,400 in the roll tube vials after appropriate incubation time. Colony diameter in the six-well plate and roll tube with dilution series having the lowest number of colonies was measured using a ruler or a slide caliper immediately after CFU was determined.

**Six-well plate cultivation with aerobic inoculation**

The volume of each well of the six-well plate (tissue culture test plate; TPP, Trasadingen, Switzerland) used in this study is approximately 15.5 mL. Eighty milliliters of W medium containing powdery gellan gum (0.45 or 0.6%, w/v), designated as W gellan medium, was dispensed into a 120-mL glass vial and degassed with N₂/CO₂ gas. W gellan medium containing 0.45% gellan gum was used for mesophiles (*D. vulgaris* and *M. bourgensis*), while that containing 0.6% was used for thermophiles (*D. thermosarovorans* and *M. thermautotrophicus*). Substrates were added to the medium at the concentrations shown in Table 1 before autoclaving at 121°C for 15 min.

The procedure of the six-well plate method with aerobic inoculation is briefly illustrated in Fig. 1. Autoclaved W gellan medium was mixed well and stored at 55°C (step 1). Reductants were added to molten W gellan medium, and then kept at 45°C for mesophiles or 55°C for thermophiles until pouring into the six-well plate. The maintenance of these temperatures prevented media from solidifying (step 2). Each well of the six-well plate was filled aerobically (on a clean bench) with about 13 mL reduced W gellan medium containing 0.6% was used for thermophiles (8:2, v/v); L, lactate (mM); M, methanol (%); NaCl (%); Pr, propionate (mM); Py, pyruvate (mM); S, sucrose (mM); SO₄²⁻ (mM); T, tryptone (%); TMA, trimethylamine (mM); Y, yeast extract (%).

<table>
<thead>
<tr>
<th>Organism</th>
<th>DSMZ number</th>
<th>Substrate (mM or %)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Methanobacterium formicicum</em></td>
<td>1535T</td>
<td>H₂, F (20)</td>
<td>37</td>
</tr>
<tr>
<td><em>Methanothermobacter thermautotrophicus</em></td>
<td>1053T</td>
<td>H₂</td>
<td>55</td>
</tr>
<tr>
<td><em>Methanoculleus bourgensis</em></td>
<td>3045T</td>
<td>H₂, A (10), F (10), Y (0.1), T (0.1)</td>
<td>37</td>
</tr>
<tr>
<td><em>Methanococcus van nielli</em></td>
<td>1224T</td>
<td>H₂, A (10) Y (0.01) NaCl (0.6)</td>
<td>37</td>
</tr>
<tr>
<td><em>Methanospiillum hungatei</em></td>
<td>864T</td>
<td>H₂, A (10)</td>
<td>37</td>
</tr>
<tr>
<td><em>Methanosarcina Barkeri</em></td>
<td>800T</td>
<td>H₂, A (20), F (20)</td>
<td>37</td>
</tr>
<tr>
<td><em>Methanococcus thermophilus</em></td>
<td>2373T</td>
<td>H₂, A (10), F (10), Y (0.1), T (0.1)</td>
<td>55</td>
</tr>
<tr>
<td><em>Methanosarcina therma phila</em></td>
<td>2905</td>
<td>H₂, M (0.5)</td>
<td>55</td>
</tr>
<tr>
<td><em>Methanosaeta thermophilid</em></td>
<td>6194T</td>
<td>A (20)</td>
<td>55</td>
</tr>
<tr>
<td><em>Methanomethylovora thermophila</em></td>
<td>17232T</td>
<td>TMA (20), Y (0.02)</td>
<td>50</td>
</tr>
<tr>
<td><em>Methanolobus taylorii</em></td>
<td>9005T</td>
<td>TMA (20), Y (0.1), T (0.1), NaCl (0.3)</td>
<td>37</td>
</tr>
<tr>
<td><em>Desulfovibrio vulgaris subsp. vulgaris</em></td>
<td>644T</td>
<td>L (20), SO₄²⁻ (10), Y (0.1)</td>
<td>37</td>
</tr>
<tr>
<td><em>Thermodesulfobacterium commun e</em></td>
<td>2178T</td>
<td>L (20), SO₄²⁻ (10)</td>
<td>55</td>
</tr>
<tr>
<td><em>Thiobacillus haffeniense</em></td>
<td>10664T</td>
<td>Py (20) SO₄²⁻ (20), Y (0.01)</td>
<td>37</td>
</tr>
<tr>
<td><em>Desulfotomaculum thermosarovor ans</em></td>
<td>6562T</td>
<td>E (20), SO₄²⁻ (10)</td>
<td>55</td>
</tr>
<tr>
<td><em>Desulfotomaculum nigrifrons</em></td>
<td>574T</td>
<td>L (20), SO₄²⁻ (10), Y (0.02)</td>
<td>55</td>
</tr>
<tr>
<td><em>Syntrophomonas wolfei subsp. wolfei</em></td>
<td>2245BT</td>
<td>C (10), Y (0.02), B (2)</td>
<td>55</td>
</tr>
<tr>
<td><em>Syntrophothermus lipocalid us</em></td>
<td>12680T</td>
<td>C (10)</td>
<td>55</td>
</tr>
<tr>
<td><em>Thermacetogenium phaeum</em></td>
<td>12270T</td>
<td>Py (20), Y (0.02)</td>
<td>55</td>
</tr>
<tr>
<td><em>Tepidanaerobacter syntrophicus</em></td>
<td>15584T</td>
<td>S (20), Y (0.02)</td>
<td>55</td>
</tr>
<tr>
<td><em>Syntrophobacillus marinosus</em></td>
<td>10017T</td>
<td>Pr (10), Fm (30), Y (0.02)</td>
<td>37</td>
</tr>
</tbody>
</table>

* A, acetate (mM); B, bromoethane sulfonate (mM); C, crotonate (mM); E, ethanol (mM); F, formate (mM); Fm, fumarate (mM); H₂, H₂/CO₂ (8:2, v/v); L, lactate (mM); M, methanol (%); NaCl (%); Pr, propionate (mM); Py, pyruvate (mM); S, sucrose (mM); SO₄²⁻ (mM); T, tryptone (%); TMA, trimethylamine (mM); Y, yeast extract (%).

*0.1% gellan gum was used for plate cultivation.

### Table 1. Anaerobic microorganisms used in this study with their cultivation conditions
Plate Cultivation of Obligate Anaerobes

Six-well plate cultivation with anaerobic inoculation

In this procedure, all steps were carried out in the same manner as for aerobic inoculation, except that steps 3 to 6 were performed in the anaerobic chamber. After step 6, the nylon bag was unzipped as little as possible to allow a gas injection needle to be inserted and the atmosphere within the bag was subsequently exchanged for the desired gases. All plastic equipment used for anaerobic inoculation was placed into the anaerobic chamber at least 1 d before the experiment to decrease the effect of oxygen contamination.

Roll tube cultivation

The roll tube method was performed according to the procedures described by Hungate and Macy (9) with slight modifications. A two-fold concentration of W medium (2×W medium) without NaHCO$_3$ was prepared in a glass vial and degassed with N$_2$/CO$_2$ gas. The vial was sealed and autoclaved at 121°C for 15 min. After autoclaving, sterilized and degassed substrates for each microorganism were subjected to 2×W medium twice as thick as the final concentrations shown in Table 1. NaHCO$_3$ solution degassed by N$_2$ gas was sterilized with a 0.2-μm pore membrane filter and added to the 2×W medium at 60 mM final concentration. Two-fold W medium was reduced with reductants and incubated at 55°C. Noble agar (BD, Tokyo, Japan) was washed with water several times and suspended in water to make approx. 4% concentration. Five milliliters of agar suspension was dispensed into 70-mL glass vial, degassed with N$_2$/CO$_2$ gas, and stored at 55°C after autoclaving at 105°C for 1 min. The reduced and preincubated 2×W medium (5 mL) was injected into the molten agar, mixed well, and incubated at 45°C for mesophiles and 55°C for thermophiles before inoculation. The same culture dilutions (0.2 mL) as those used for the six-well plate method were inoculated into the molten agar media with needles and syringes, and then the vials were rolled on ice to make roll tubes. For methanogens, the gas phase of the vial was replaced and pressurized with H$_2$/CO$_2$ at approx. 0.15 MPa after solidification of agar.

Six-well plate cultivation of anaerobes

The six-well plate cultivation method with aerobic inoculation was applied to the microorganisms listed in Table 1 according to the procedures described above. Cultures were taken by syringes and needles from vials into 1.5-mL plastic tubes, and then diluted with reduced W medium on a clean bench. Growth substrates and incubation temperatures for each microorganism are listed in Table 1. A single colony was sucked out from the six-well plate with a 25-gauge needle and a 1-mL syringe, and then inoculated directly into fresh liquid medium on the clean bench. Microorganism growth was confirmed by culture turbidity.

Isolation of an obligate anaerobe from an environmental sample using six-well plate method with aerobic inoculation

Gas-associated formation saline water from a gas-water-producing well in Niigata, Japan was used to isolate an obligate anaerobe using the six-well plate method. A water sample from the well was collected at a depth of 1,000 m below the land surface. The temperature and pH of the sample water were 41°C and 7.4, respectively. A sulfate-reducing bacterium (SRB) was targeted for isolation from the sample. W medium was modified with MgCl$_2$·6H$_2$O (15 mM, final concentration), Na$_2$SO$_4$ (20 mM), NaCl (350 mM), and Na$_2$S·9H$_2$O (0.36 g L$^{-1}$) as a reducing agent, and H$_2$/CO$_2$ (ca. 0.15 MPa), which is hereafter referred to as WS medium. SRB was enriched in WS medium using the formation water as an inoculum. After several passages of subculture at 45°C, enrichment was applied to the six-well plate method with aerobic inoculation. Gellan gum medium was modified on the basis of the WS medium ingredients (WS gellan medium), and the concentrations of gellan gum, MgCl$_2$·6H$_2$O, and NaCl were reduced to 0.3%, 6 mM, and 175 mM, respectively, since the high salinity of medium caused its rapid solidification. WS gellan medium after autoclaving was kept at 70°C until inoculation. Serial dilutions of SRB enrichment culture were prepared with the reduced WS medium and inoculated (0.1 mL) into wells containing unsolidified WS gellan medium. The plate within the bag was incubated at 45°C after gas exchange with
H₂/CO₂. Colonies were picked and transferred into the same medium in the vial as used for the enrichment culture. The PCR product of 16S rRNA gene was directly sequenced as previously described (13). The obtained sequence was analyzed with sequences deposited in a public database using BLAST (1) at the website of the National Center for Biotechnology Information. The isolated SRB, designated strain KNH, was deposited in NBRC as NBRC 107642.

**Analytical Methods**

Gas phase analysis was performed in a vial with a GC-8A gas chromatograph (Shimadzu, Kyoto, Japan) as previously described (17). Tukey’s HSD test was performed with KyPlot 4.0 (KyensLab, Tokyo, Japan) for comparison of CFUs among different methods. Microphotographs were obtained using a phase contrast microscope (BX51; Olympus, Tokyo, Japan) equipped with a digital CCD camera (DP71; Olympus).

**Nucleotide sequence accession numbers**

The 16S rRNA gene sequence of isolated SRB has been deposited in the DDBJ/EMBL/GenBank database under accession number AB518055.

**Results**

**Comparison of cultivation efficiency between six-well plate and roll tube cultivation methods using representative anaerobes**

The cultivation efficiency of the examined species is summarized in Table 2. CFU was converted into a value relative to that of the roll tube. For both *M. bourgensis* and *D. vulgaris*, the differences in CFUs among the three methods were not significant (*P*=0.05). For *M. thermototrophicus*, the CFU of the six-well plate method with anaerobic inoculation was among the highest and showed significant differences from that of six-well plate method with aerobic inoculation (*P*<0.05) and from the roll tube method (*P*<0.01). In the case of *D. thermonovorans*, CFUs in the six-well plate methods with both anaerobic and aerobic inoculations were 75 and 41 times higher than that of the roll tube method, respectively. Anaerobic inoculation gave rise to higher CFU than aerobic inoculation (*P*<0.01). The incubation time required for visible colony formation for *M. thermototrophicus* and *D. vulgaris* was equal in all methods. Colonies in the roll tube of *M. bourgensis* were confirmed after 17 d of incubation, while those in both six-well plates were found after 12 d. With *D. thermonovorans*, colonies were found 6 d in the roll tube method, while 3 d in both six-well plate methods. Changes to CFUs in the roll tube cultures of these organisms were further determined after an extended period of incubation (up to 3 months), but no additional colonies emerged. When similar numbers of colonies were found in the roll tube and six-well plate, the maximum diameters of colonies were generally larger in the vials of the roll tube method than the wells of the six-well plate methods (see Table 2). Moreover, similar diameters of colonies were found in the wells of both six-well plate methods where similar numbers of colonies occurred (*M. bourgensis*, *D. thermonovorans*, and *D. vulgaris*), showing that the presence or absence of oxygen during inoculation into the plate had no effect on the diameter of colonies.

Further experiments using the six-well plate method were performed with aerobic inoculation, since we concluded that both anaerobic and aerobic inoculations gave similar degrees of cultivation efficiency.

**Six-well plate cultivation of anaerobes**

The ability of obligate anaerobes to grow using the six-well plate method was further examined with 11 methanogens (7 hydrogenotrophic, 1 acetoclastic, and 3 methylotrophic), 4 SRB, 1 thiosulfate-reducing bacterium, and 5 *H₂*-producing syntrophs. Colony formations of all examined strains were confirmed by the six-well plate method, and subcultures of these colonies into fresh liquid media were successful. Among many obligate anaerobes, *Methanosaeta* spp. are known to be fastidious organisms on solid media, hence they have been isolated using dilution-to-extinction methods in liquid medium (10, 12, 15). In fact, the first attempts to cultivate *Methanosaeta thermophila* by the six-well plate method failed when using 0.6% gellan gum medium. Carbonero et al. (5) have recently found that a low concentration of gellan gum (0.1%) is suitable for cultivation of *Methanosaeta* spp., so

<table>
<thead>
<tr>
<th>Organism</th>
<th>Roll tube</th>
<th>Plate aerobic inoculation</th>
<th>Plate anaerobic inoculation</th>
<th>Roll tube</th>
<th>Plate aerobic inoculation</th>
<th>Plate anaerobic inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Methanothermobacter thermototrophicus</em></td>
<td><em>(9.5±2.7)×10⁷</em></td>
<td><em>(1.5±0.2)×10⁸</em></td>
<td><em>(3.3±0.1)×10⁸</em></td>
<td>1</td>
<td>1.6</td>
<td>3.5</td>
</tr>
<tr>
<td><em>Methanoculleus bourgensis</em></td>
<td><em>(5.6±0.4)×10⁷</em></td>
<td><em>(5.2±1.6)×10⁷</em></td>
<td><em>(6.3±0.8)×10⁷</em></td>
<td>1</td>
<td>0.93</td>
<td>1.1</td>
</tr>
<tr>
<td><em>Desulfotomaculum thermonovorans</em></td>
<td><em>(1.6±0.1)×10⁵</em></td>
<td><em>(6.5±1.2)×10⁶</em></td>
<td><em>(1.2±0.1)×10⁷</em></td>
<td>1</td>
<td>41</td>
<td>75</td>
</tr>
<tr>
<td><em>Desulfovibrio vulgaris</em></td>
<td><em>(3.8±0.1)×10⁶</em></td>
<td><em>(4.0±0.6)×10⁹</em></td>
<td><em>(4.4±1.2)×10⁸</em></td>
<td>1</td>
<td>1.1</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* CFUs are expressed as avg±SD (*n*=3). The number of colonies was determined after 9, 17, and 28 d of incubation for *M. thermototrophicus* and *D. vulgaris*, *D. thermonovorans*, and *M. bourgensis*, respectively.
we decreased the gellan gum concentration to 0.1% and eventually obtained colonies. Sulfate- or thiosulfate-reducing bacteria could also develop colonies in the six-well plate. Of the tested syntrophs, *Syntrophomonas wolfei* subsp. *wolfei* was purchased and maintained in coculture with *Methanospirillum hungatei* as an H₂-scavenging methanogenic partner using butyrate as a substrate. For isolation of the organism by separating it from partner methanogen, bromoethane sulfonate (as methanogen inhibitor) and crotoenate (known as a fermentative substrate allowing *S. wolfei* subsp. *wolfei* to grow in pure culture) were added to medium (3). Pure culture of *S. wolfei* subsp. *wolfei* was eventually obtained and its purity was confirmed by microscopy after cultivation of inoculated colonies in liquid medium; thus, separation and isolation (pure culture) of a syntroph from methanogenic syntrophic culture were shown to be possible using the six-well plate method.

**Isolation of obligate anaerobes from environmental sample using six-well plate method**

We previously examined microbial diversities on gas-associated formation subsurface water and found that the sample contained an uncultured SRB revealed by 16S rRNA gene clone library analysis (Mochimaru, unpublished data). An enrichment culture of SRB was made from the sample and maintained in WS medium. Colonies were successfully developed in the six-well plate and several colonies were separately inoculated in liquid culture. After two purifications with the six-well plate method (3 weeks each of incubation at 45°C), an organism designated strain KNH, forming gray (dark in center), round, and flat colonies of approx. 4 mm in maximum diameter, was eventually obtained within gellan gum medium (Fig. 2A). The sequence analysis of the 16S rRNA gene (1,642 bp) revealed that strain KNH is distantly related to *Desulfomaculum kuznetsovii* strain 17 (22) and *Candidatus ‘Desulforudis audax viator’ MP104C* (6) with 86% and 92% identities, respectively. Cells of strain KNH after 40 d of cultivation under a phase contrast microscope were curved rods, 2.5–9.7×0.6 μm in size, and some had swollen termini, implying spore formation (Fig. 2B).

**Discussion**

Cultivation and isolation still have indispensable roles in microbiology, although next-generation sequencing techniques have enabled us to analyze the functions of microbes in the ecosystems without their cultivation (14, 25). Information obtained from isolates reveals not only their functions *in vitro* but also their potent physiologies in the environment. In addition, this could be applied to metagenomic data derived from next-generation sequencing techniques. The importance of cultivation and isolation is being reacknowledged; however, the culture of obligate anaerobes tends to be limited because the special apparatus and careful techniques required make it laborious. Therefore, we have developed a simple and effective culturing method using easily-available, easy-to-use, and disposable plasticware for diverse uncultured obligate anaerobes in the environment.

The cultivation efficiency of the six-well plate methods was first evaluated in comparison with the roll tube method. We found that the highest CFUs were obtained with the six-well plate method with anaerobic inoculation for all examined representative samples. There is no doubt that highly anaerobic conditions for manipulation give rise to the highest CFU, as this procedure minimizes the exposure of obligate anaerobes to oxygen; however, there were no significant differences between aerobic and anaerobic inoculations of *M. bourgensis* and *D. vulgaris*, as a result of their aerotolerance. The examined strain of *D. vulgaris* (strain Hildenborough) is known as an oxygen-resistant sulfate reducer and is even capable of forming ATP under microaerophilic conditions (4). Although the aerotolerance of *M. bourgensis* is unknown, genomic information on its closely related species (*M. marisnigri*) implies its resistance to oxygen (reactive oxygen species), because of conserved genes encoding antioxidant enzymes, such as superoxide dismutase, peroxiredoxins, and peptide methionine sulfoxide reductases (2). These enzymes might also exist in *M. bourgensis*. Compared with the six-well plate method with aerobic inoculation, the roll tube method seems to involve less exposure of cells to oxygen; however, some organisms gave rise to many fewer CFUs with the roll tube method. In particular, in the case of *D. thermosapovorans*, the six-well plate method with aerobic inoculation had 41 times greater CFU than the roll tube method. The reason for this remains to be clarified, but one plausible explanation is the difference in the gelling agent. In fact, gellan gum is reported as a superior gelling agent to agar in terms of CFU when applied to the cultivation of some methanogens and aerobic bacteria (8, 11, 20, 21). It is implied that gellan gum contains fewer inhibitory substances for microbial growth than agar (8). We attempted to make roll tubes using gellan gum to determine...
its effect on CFU; however, all attempts were unsuccessful as gellan gum quickly solidified during the tube-rolling process. A decrease in the concentration of gellan gum to prevent rapid solidification caused a lack of firmness of the medium, resulting in the medium slipping from the wall of the tube. Indeed, gellan gum is not an easy solidifying agent to handle compared with agar, especially in the roll tube method. Meanwhile, agar was examined as a gelling agent for the plate method; however, the turbidity of agar thickly poured into wells impaired the visibility of colonies so we decided to use gellan gum as an appropriate gelling agent for the plate method. Differences in the gelling agents might also result in differences in cultivation time for the development of visible colonies of M. bourgensis and D. thermosapovorans. Differences in the sizes of colonies occurred on the six-well plates and roll tubes, probably derived from the difference in surface areas between them, especially in the cases of M. thermautotrophicus and M. bourgensis, which live on a gaseous substrate (H₂/CO₂). The far larger surface area of the medium in a roll tube is thought to lead to higher gas exchange than in a well, so that colonies in a roll tube become larger than those in wells during the same incubation period. The roll tube method resulted in a larger colony size for some hydrogenotrophic anaerobes; however, we are not always able to obtain such large colonies (≥2 mm diameter). Small colonies (<1 mm diameter) must be carefully picked up from roll tubes and require careful techniques using a Pasteur pipette with a bent tip and a mouse tube (9). In contrast, those on the six-well plate are relatively easily selected because they can be viewed clearly and are highly accessible with syringes and needles. Based on these findings, we concluded that six-well plate method is superior to the roll tube method in terms of the manipulation of small colonies. Thus, these results evidently show that the cultivation efficiency of the six-well plate methods with either aerobic or anaerobic inoculation was comparable or even superior to that of the roll tube method. All examined anaerobes developed colonies using the six-well plate method with aerobic inoculation and their colonies succeeded in subcultures within liquid media. Moreover, the six-well plate method with aerobic inoculation enabled us to obtain an uncharacterized SRB strain from the environmental sample. As shown from its isolation, optimization of the concentration of salt and/or gellan gum in the six-well plate method may become a solution for the isolation of obligate anaerobes in high salinity environments, except halophiles. These results strongly suggest that our new method is applicable for the cultivation and isolation of a wide variety of yet-to-be-cultured anaerobes in ecosystems. In this context, we conclude that the six-well plate method is a simple, less laborious, and alternative method for the cultivation and isolation of obligate anaerobic microorganisms.

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