Specific Antisera and Immunological Procedures for Characterization of Methanogenic Bacteria

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Specific antisera were raised in rabbits to 19 methanogenic bacteria representing the species available in pure culture at the present time. The antisera were characterized, labeled, and organized in a bank to serve as a source of material for preparation of antibody probes and thus provide standardized reagents for immunological analysis of methanogens. An indirect immunofluorescence procedure was standardized for optimal staining of homologous and heterologous bacterial strains. Two immunoenzymatic assays were developed: (i) a simple slide assay, useful for rapid antibody detection in small samples, antibody titrations, and disclosure of cross-reactions among methanogens, and (ii) a quantitative method. The latter is useful for quantification of antigenic relatedness. Procedural details were developed to obtain optimal bacterial preparations for use as immunogens to raise antibodies in vivo, and as antigens for antibody assay in vitro.

Methanogenic bacteria or methanogens are a diverse group of microorganisms that share the unique property of producing methane (1, 33). The features that distinguish methanogens from eubacteria include differences in cell walls, lipids, rRNA’s, and coenzymes that are unique to methanogens (1-3, 7-10, 12-16, 20, 23, 25, 26, 29, 33). The methanogens themselves are a diverse group and have been placed in different species, genera, and families on the basis of physiology, morphology, cell walls, and 16S rRNA’s (1, 8, 30-32). Other than examining morphological variations and determining a few differences in physiological characteristics, methods for distinguishing between methanogens rely upon analyses of differences in macromolecules, particularly 16S rRNA. Although determination of the composition of macromolecules provides the most definitive information about degree of relatedness between different methanogens, the analytical methods are not suitable for rapid identification of these bacteria. Immunological methods offer a simpler and quicker alternative, have been useful for identification of other groups of bacteria, and are an important complement to biochemical procedures. Moreover, differences in cell wall composition (12-16) suggest that methanogens carry distinctive surface markers capable of eliciting antibodies potentially useful for identification and classification purposes (24, 28; E. Conway de Macario, M. J. Wolin, and A. J. L. Macario, Fed. Proc. 39:677, 1980; E. Conway de Macario, A. J. L. Macario, A. E. Alito, and M. J. Wolin, Fed. Proc. 40:1594, 1981; 5a). In this report we describe immunological probes and procedures for the study of methanogens and demonstrate how they can be used to identify these bacteria and to detect antigenic relationships among them.

MATERIALS AND METHODS

Cultures. Antisera were prepared against the bacteria listed in Table 1, which also indicates the investigators who kindly supplied the cultures. Two strains were obtained in pure culture in our laboratory, Methanobacterium thermoautotrophicum GC1 and Methanobrevibacter smithii ALI. GC1 was isolated from an enrichment culture that was maintained at 60°C on primary sludge from a sewage treatment plant. ALI was isolated from an in vitro semicontinuous culture of the microbial community of human feces (21a). GC1 had the same morphological and physiological characteristics as M. thermoautotrophicum, and ALI had the same morphological and physiological characteristics as M. smithii (1).

Media. The media used to grow cells for the preparation of antigen suspensions are listed in Table 1. M1 medium was the same as previously described (4). The media designated as Balch media were the same medium 1 (Balch 1) and medium 3 (Balch 3) of Table 1 of Balch et al. (1), except that the composition of the trace vitamin solution was different. We used, per liter of medium, 10 ml of a solution of vitamins that contained 20 mg each of thiamine HCl, nicotinamide, riboflavin, pyridoxin HCl, and calcium D-pantothenate, 2 mg of cyanocobalamin, 10 mg of biotin, 1 mg of p-aminobenzoic acid, and 0.5 mg of folic acid per 100 ml of distilled water. The cysteine-sulfide solution (4)
was added aseptically to sterile media. Balch 1m was the same as Balch 1 with the addition of 2-mercaptoethanesulfonic acid and the volatile fatty acid supplement and modification of the NaHCO₃ concentration described by Balch et al. for cultivation of *Methanobrevibacter ruminantium*. Balch 1 + NH₄Cl was Balch 1 with an additional 1 g of NH₄Cl per liter of medium. E1 medium was a minor modification of a medium devised by Mah (personal communication). E1 contained the following per liter: NH₄Cl, 1 g; K₂HPO₄, 0.3 g; MgCl₂·6H₂O, 0.1 g; yeast extract, 2 g; tryptcase, 2 g; cysteine-HCl·H₂O, 0.5 g; NaHCO₃, 0.5 g; and resazurin, 0.001 g. After dissolving all ingredients except cysteine and NaHCO₃, the pH was adjusted to 6.6 to 6.8 with 10 N NaOH, and the solution was heated to boiling and then rapidly cooled while gassing with O₂-free N₂. Cysteine and NaHCO₃ were added, and 49 ml of medium was dispensed in 100-ml serum bottles with continuous flushing with N₂. Bottles were capped with butyl rubber stoppers as they were removed from the gassing probe, sealed with aluminum caps, and sterilized by autoclaving. Before inoculation, 1 ml of a sterile 20% solution of methanol was added to the medium. The methanol solution was prepared in serum bottles in an N₂ atmosphere before sterilization by autoclaving. E2 medium was the same as E1 medium except 300 ml of clarified rumen fluid was added per liter before dispensing the medium in 48-ml amounts in serum bottles, cysteine was omitted, and 0.5 ml each of 1% Na₂S·9H₂O and 1% CaCl₂ and 1 ml of 2 M sodium acetate were aseptically added to each serum bottle before inoculation. The latter three solutions were sterilized separately in serum bottles under N₂.

**Growth of organisms.** Stock cultures were generally maintained on the media described for each organism in Table 1. Media used for preparing inocula for production of cells for antigen preparations were also generally the same. Details relating to the maintenance of individual cultures, preparation of inocula, and amounts of inocula can be obtained from the authors upon request. Anaerobic techniques were essentially

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TABLE 1. Antiserum bank

<table>
<thead>
<tr>
<th>Antiserum*</th>
<th>Species and strain</th>
<th>Source*</th>
<th>Temp (°C)</th>
<th>Atm</th>
<th>H₂:CO₂</th>
<th>N₂</th>
<th>Medium*</th>
</tr>
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<tbody>
<tr>
<td>Anti-MF (RI661)</td>
<td><em>Methanobacterium formicicum</em> MF</td>
<td>MPB</td>
<td>37</td>
<td>3</td>
<td>50:50</td>
<td>M1</td>
<td></td>
</tr>
<tr>
<td>Anti-MoH (RL649)</td>
<td><em>M. bryantii</em> MoH</td>
<td>RSW</td>
<td>37</td>
<td>2</td>
<td>80:20</td>
<td>M1</td>
<td></td>
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<tr>
<td>Anti-MoHG (RI926)</td>
<td><em>M. barkeri</em> MoHG</td>
<td>RSW</td>
<td>37</td>
<td>2</td>
<td>80:20</td>
<td>M1</td>
<td></td>
</tr>
<tr>
<td>Anti-AH (RN382)</td>
<td><em>M. thermoautotrophicum</em> ΔH</td>
<td>RSW</td>
<td>60</td>
<td>2</td>
<td>80:20</td>
<td>Balch 1d</td>
<td></td>
</tr>
<tr>
<td>Anti-GC1 (RN266)</td>
<td><em>M. thermoautotrophicum</em> GC1</td>
<td>MJW</td>
<td>60</td>
<td>2</td>
<td>80:20</td>
<td>M1</td>
<td></td>
</tr>
<tr>
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<td>RSW</td>
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<td>2</td>
<td>80:20</td>
<td>Balch 1m</td>
<td></td>
</tr>
<tr>
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<td><em>M. arborophilus</em> DH1</td>
<td>RSW</td>
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<td>80:20</td>
<td>Balch 1</td>
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<td>Anti-PS (RI660)</td>
<td><em>M. smithii</em> PS</td>
<td>MPB</td>
<td>37</td>
<td>3</td>
<td>50:50</td>
<td>M1</td>
<td></td>
</tr>
<tr>
<td>Anti-ALI (RN265)</td>
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<td>37</td>
<td>2</td>
<td>80:20</td>
<td>M1</td>
<td></td>
</tr>
<tr>
<td>Anti-SB (RN302)</td>
<td><em>Methanococcus</em> vannielli SB</td>
<td>RWS</td>
<td>28</td>
<td>2</td>
<td>80:20</td>
<td>Balch 3</td>
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</tr>
<tr>
<td>Anti-PSv (RN303)</td>
<td><em>M. voltae</em> PSv</td>
<td>RWS</td>
<td>28</td>
<td>2</td>
<td>80:20</td>
<td>Balch 3</td>
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<td>80:20</td>
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<td></td>
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<td><em>Methanospirillum hungatei</em> JF1</td>
<td>MPB</td>
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<td>2</td>
<td>80:20</td>
<td>M1</td>
<td></td>
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<td>M1</td>
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<td><em>M. barkeri</em> 227</td>
<td>RAM</td>
<td>37</td>
<td>2</td>
<td>80:20</td>
<td>Balch 1d</td>
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<tr>
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<td>MPB</td>
<td>37</td>
<td>3</td>
<td>80:20</td>
<td>M1</td>
<td></td>
</tr>
<tr>
<td>Anti-W (RN660)</td>
<td><em>M. barkeri</em> W</td>
<td>RAM</td>
<td>37</td>
<td>1</td>
<td>100</td>
<td>E1</td>
<td></td>
</tr>
<tr>
<td>Anti-MC-6 (RN648)</td>
<td><em>Methanococcus mazei</em> MC6</td>
<td>RAM</td>
<td>37</td>
<td>1</td>
<td>100</td>
<td>E2</td>
<td></td>
</tr>
</tbody>
</table>

* Each antiserum was defined by the rabbit letter and number, bleeding serial number and date, and vial letter as described in the text. Data in this paper were obtained using serum from the first bleeding vial a. For simplicity, only rabbit letter and number are shown in this table.

* We thank M. P. Bryant (MPB), R. S. Wolfe (RWS), and R. S. Mah (RAM) for making their strains available to us. Strains *M. smithii* ALI and *M. thermoautotrophicum* GC1 were isolated in our laboratory (M. J. Wolin [MJW]). Large-scale growth was done in all cases in our laboratory (MJW).

* For description of the media used, see text and references 1 and 19.

* Balch + NH₄Cl.

* Lower case letters v, c, and m were not used originally for strains *M. voltae*, *M. cariaci*, and *M. marisnigri*, respectively. The letters were added to us to avoid confusion (e.g., between *M. smithii* PS and *M. voltae* PS).
as described previously (20). The gas atmospheres and temperatures used for growth of cells for antigen preparations are shown in Table 1. Where H₂CO₂ mixtures were used, the medium was prepared under 50% H₂–50% CO₂. After inoculation the bottles were pressurized to the indicated pressure (Table 1). For 80% H₂–20% CO₂ the bottles were flushed after inoculation with the H₂–CO₂ mixture with an inlet syringe plus hypodermic needle probe and an outlet hypodermic needle. After flushing for 1 to 2 min, the exit needle was removed and the bottles were pressurized to the indicated pressures (Table 1). Black butyl rubber stoppers (Bellco Glass, Inc., Vineland, N.J.) were used for pressurized bottles. Cultures were incubated until the optical density at 660 nm (OD₆₆₀, 1-cm light path) was 0.5 to 0.8 when measured against a water blank. H₂–CO₂ cultures were incubated on a shaker and were regassed and repressurized if the gas pressure was 1 atm or less before the culture reached the above-mentioned OD.

**Bacterial suspensions.** Fixed stock suspensions were prepared by harvesting by centrifugation at 12,000 × g for 10 min. The supernatant was discarded, and the pellet was suspended in a solution containing 8.5 g of NaCl per liter and 4% (vol/vol) Formalin. Salt concentration was increased to 1.8% for suspension of *M. ruminantium* M1. After adjusting the suspension to an OD₆₆₀ of 0.5 (1-cm light path), it was stored at 4°C for use when necessary for immunization and preparation of smears for immunofluorescence (IF). Printed glass slides (J. Melvin Freed, Inc., Perkasie, Pa.) were used for bacterial smears, the volume and concentration of which are described below.

**Rabbits, immunization, and collection of sera.** Rabbits were supplied by the Griffin Laboratory (Division of Laboratories and Research, New York State Department of Health, Albany, N.Y.).

A 1.5-ml portion of the formalized suspension was centrifuged at 12,000 × g for 10 min. The supernatant was discarded, and the pellet was suspended in 10 ml of phosphate-buffered saline (PBS), pH 7.2. Centrifugation and suspension were repeated twice. After a third centrifugation, the pellet was resuspended in 0.7 or 1 ml of PBS for primary or secondary immunization, respectively. Smears were prepared with 20 μl of these suspensions to check the morphology of the organisms and also their antigenicity by IF whenever the corresponding antiserum was available. The remaining suspension was emulsified in an equal volume of Freund complete adjuvant. A priming dose was given in one rear footpad, and a boosting dose was administered 28 days later subcutaneously in three different spots of the neck (5). Rabbits werebled from the central ear artery 14 and 28 days after the boosting.

**Organization of the serum bank.** Forty to 50 ml of serum was obtained at each bleeding and stored in 5-ml vials at −70°C. Each serum was identified with rabbit designation, bleeding number, and date and vial serial letter: e.g., RL651, 1st (4–16–79), a. A protocol was kept for each rabbit in which all immunizations, bleedings, etc., were recorded in chronological order, together with information on the bacterial strain used for immunization: designation, source of inoculum, culture conditions, batch, etc. Additional information on the microorganisms was recorded in a separate form for each isolate.

A single batch of stock bacterial suspension was used for the complete immunization schedule of each rabbit. The serum vial in use was split into 1-ml aliquots and kept at 4°C. All operations were carried out with syringe and needle under sterile conditions. The results reported in this paper were obtained with the first bleedings, vial a, from each rabbit and immunizing strain.

**Antibody measurements: IF.** The indirect IF technique (5a, 11, 18) was used. Heat-fixed bacterial smears were incubated in a humid chamber at 23°C with the anti-methanogen antiserum or the control serum (from a normal rabbit or from a rabbit inoculated with Freund complete adjuvant) and then washed and incubated with the labeled anti-rabbit gamma globulin antisera and washed again. The standard incubation time with both antisera was 45 min. Washings were performed by dropwise addition of 10 ml of PBS on each slide followed by its immersion in a beaker containing PBS for 10 min. Afterwards, washing and immersion procedures were repeated with distilled water. Finally, the slides were dried with warm air. Goat anti-rabbit gamma globulin antisera labeled with fluorescein isothiocyanate (fluorescein/protein ratio, 3 mg/g) was purchased from ICN Pharmaceuticals, Inc., Cleveland, Ohio. This antiserum was calibrated to determine the optimal dilution in PBS to be used by assaying a series of twofold dilutions with a series of dilutions of an anti-methanogen antiserum. The optimal dilutions were either 1:25 or 1:50 depending on the protein concentration and batch of the anti-antiserum. Calibration was repeated with each batch several times a year to ensure the reproducibility of results obtained with the same reagents in tests far apart in time. No significant variations were found over periods of 8 months.

The reactions of antigens with an antiserum and a control serum were performed simultaneously in adjacent rings of the same slide. Controls included the following: serum from a normal uninfected rabbit, serum from a rabbit injected with Freund complete adjuvant substituted for anti-methanogen antiserum, omission of all sera and of the anti-methanogen antiserum, and omission of the labeled anti-rabbit gamma globulin antisera. These controls never showed fluorescence.

The slides were coded and read using a Zeiss microscope (narrow band exciter filter BP 485/20 nm, light source HBO 50 W/L2 mercury arc) at 1,000× under oil. Reactions were graded from 1+ to 4+ according to the intensity of the fluorescence. Photographs were taken with Kodak Ektachrome 400 ASA film (Eastman Kodak, Co., Rochester, N.Y.).

**Quantitative immunoenzymatic assay.** Twenty-five μl of PBS containing the appropriate number of formalized bacteria was added to a disposable glass test tube (12 by 75 mm). Vacuum tubing was inserted halfway down the tubes, which were warmed over a flame. Heat and vacuum were applied until the cell suspension dried on the bottom of the tube. Bacteria were further heat fixed by passing the tube bottom through a flame three times. After cooling to room temperature, salts were gently rinsed away with glass-distilled water. The tubes were then flushed with 95% ethanol to hasten drying and enhance cell fixation. Fifty microliters of antibody sample or control solution was added, and the tubes were plugged with
rubber stoppers and gently agitated. After incubation at 23°C in a dark chamber for 30 min to allow antibody contact, the supernatant was aspirated off with a 9-inch (ca. 23-cm) Pasteur pipette. Bacteria were washed twice with 1 ml of CM (Dulbecco modified Eagle medium, containing nonessential amino acids, L-glutamine, and 5% [vol/vol] calf serum and gamma globulin-free horse serum; GIBCO Laboratories, Grand Island, N.Y.) and then covered with 1 ml of CM for 10 min. The CM was discarded, the two washings were repeated with distilled water, and the tubes were dried under vacuum. One hundred microliters of goat immunoglobulin G (IgG) anti-rabbit IgG (or antimouse IgG) labeled with peroxidase (Cappel Laboratories, Cochranville, Pa.) at the desired dilution in CM was added to the tubes, which were then incubated at 23°C for 30 min before washing with CM and distilled water as described above. Finally, 250 μl of the substrate solution containing 0.1% of o-phenylenediamine (Aldrich Chemical Co., Milwaukee, Wis.), 98% recrystallized once, and 20% [vol/vol] H₂O₂ (J. I. Baker, Phillipsburg, N.J.) in 0.1 M citrate buffer (pH 4.5) was added to each tube. The reaction was timed, and the absorbance at 420 nm was measured in a spectrophotometer (Gilford model 250 with an enzyme-linked immunosorbent assay aspirating microcuvette model 3018-B). The readings were transformed into OD₄₂₀/min, and the mean value of the negative control tubes (sera from normal animals or from animals injected with Freund complete adjuvant) was subtracted from the value of the corresponding experimental reaction. In this way the absolute OD₄₂₀/ min (Abs OD/min) was obtained and taken as the quantitative expression of the reaction. In cases where homologous and cross-reactions were compared, the test was calibrated by choosing the concentration of the antigens and the corresponding homologous antibody dilution giving the same Abs OD/min. These concentrations of antigens and antibodies were then tested with the heterologous antibody and antigen, respectively. The cross-reaction was expressed as the percentage of the homologous reaction using the following equation: (CR Abs OD/min)/(HR Abs OD/min) × 100 = percent cross-reaction, where CR and HR stand for cross- and homologous-reaction, respectively.

(iii) Slide immunoenzymatic assay. We used printed glass slides for IF (Roboz Surgical Instrument Co. Inc., Washington, D.C.). Slides with eight circles (8 mm in diameter) were cleaned with 95% (vol/vol) ethanol and air dried. Twenty-five microliters of CM containing the desired number of formalinized bacteria was added to each circle, air dried, and heat-fixed. Twenty-five microliters of the antibody (or control fluid) dilution in CM was then placed onto each circle, and the slide was kept at 23°C in the dark, in a humid chamber for 20 min. The circles were then washed by dropwise addition of 5 ml of CM followed by immersion in CM for 10 min. The same washing procedure was then used to wash twice with distilled water, and the slides were air dried. Fifty microliters of the peroxidase-labeled goat IgG anti-rabbit (or anti-mouse as required) IgG at the desired dilution in CM was placed on each circle, incubated, washed, and air dried as described above. Finally, 50 μl of the o-phenylene diamine solution containing H₂O₂ (see above) was deposited on each circle, and the emergence of yellow color and its intensity were recorded at 5, 15, and 30 min. The intensity of the reaction was estimated by grading the color intensity from 0 (no color) up to 4+ (intense yellow). Formation of precipitates was also recorded at later times since they correlated very well with the intensity of the reaction and stayed fixed to the slide after the substrate evaporated. This afforded a simple means of keeping an accurate and stable document of the reaction for months. Negative controls, i.e., circles in which the antibody sample was replaced by normal serum, serum from animals inoculated with Freund adjuvant, or CM, were colorless and without precipitates.

RESULTS

Serum bank and antibody probes. Antisera to 19 strains of methanogens were produced in rabbits and organized in a bank of well defined records (Table 1). Each antiserum was titrated by assaying a series of twofold dilutions against the immunizing (homologous) methanogens (Fig. 1). Selected dilutions of each antiserum were then assayed with the rest of the methanogens (heterologous). It was found that the most useful dilutions (i.e., probes) for identifying methanogens and for establishing antigenic relatedness among them are the ones indicated by letters in Fig. 1. A more detailed definition and use of these probes is given in the accompanying paper (6).

Optimal volume and concentration of bacterial suspensions for smears for IF. Smears were prepared with bacterial suspensions of various concentrations and examined after completion of the IF reaction to determine the quality of the staining and density and distribution of the microorganisms. A smear was considered satisfactory if the microorganisms were evenly distributed without overlapping or aggregation that could hinder observation of individual bacteria. Experiments were performed with Methanobacterium formicicum MF as a model, since its size and morphology represent the average between coccobacilli (e.g., M. smithii PS) and long curved rods (e.g., Methanospirillum hungatei JF1). The sarcina are different since they are packets of large round bodies and were studied separately.

Bacterial suspensions of OD₆₆₀ values of 0.087 (1-cm light path) and 0.175 yielded better smears than suspensions of 0.043 and 0.350. Within the former limits, the bacteria were evenly distributed without overlapping but sufficiently close to each other to allow the observation of many of them per each microscope field at 1,000×. Their contours were sharp, and no clumps were found with the exception of M. smithii PS. This strain tended to aggregate.

A homogeneous suspension containing mostly pairs or short chains was obtained as follows: 1 ml of bacterial suspension (OD₆₆₀ of 0.100) was
aspirated 25 times from a 5-ml tube with a 1-ml syringe and a 26-gauge, 3/8-inch (ca. 0.95-cm) needle, bent at two angles. The volume of bacterial suspension to obtain smears with 7-, 9-, and 13-mm diameter circle slides was 5, 10, and 20 µl, respectively.

Optimal incubation times for IF in homologous and heterologous combinations. Incubation times with both the anti-methanogen and the fluorescein-labeled anti-immunglobulin antisera were varied to determine the shortest interval giving maximal IF, namely the time necessary for the reaction to obtain the plateau without background and nonspecific staining. The plateau was usually reached in 45 min, but in some cases the reaction of the anti-methanogen antiserum with the immunizing bacterium took only 15 min. Examples of both situations are shown in Fig. 2.

Antigenicity of formalinized stock suspensions. Since formalinized suspensions were stored for several months, it was necessary to determine whether their antigenicity decayed with time. An example of the three situations encountered is given in Fig. 3. Some methanogens (e.g., *M. formicicum* MF) did not lose antigenicity in over a year of storage, whereas others began to show impairment of antigenicity on day 100 (e.g., *Methanobacter bryantii* MoHG) or at later times (e.g., *M. smithii* PS).

Immunoenzymatic assay. The results of IF were confirmed by the slide immunoenzymatic assay, which likewise showed a reaction that could be evaluated by visual estimation of the yellow color, from 4+ (intense yellow) down to 0 (no color) (Fig. 4). The immunoenzymatic reaction was also measured spectrophotometrically, and this allowed a quantitative estimation of cross-reaction as exemplified in Table 2, where it is shown that, under the assay conditions, *Methanogenium cariaci* JRlc cross-reacts 33% with rabbit *M. hungatei* JFI antiserum.

Morphological features of the IF reaction of methanogens. Figure 5a-d shows examples of IF reaction of four major morphological forms of methanogens: cocci, rods, very long rods, and sarcina. Some strains (*M. formicicum* MF, *M. bryantii* MoH and MoHG, and *M. smithii* PS) showed different morphological phenotypes if the culture conditions were varied. However, no antigenic differences were detected among the
FIG. 4. Example of slide immunoenzymatic assay for antibody titration in a rabbit *M. hungatei* JF1 antiserum. JF1 bacteria were heat fixed to the slide and then reacted sequentially with the antiserum, goat gamma globulin anti-rabbit gamma globulin labeled with peroxidase, and lastly with substrate (see text for details). A series of twofold dilutions of the JF1 antiserum were tested (1:200 through 1:6,400) from the uppermost through the second circle in the bottom row. The last two circles were controls in which JF1 antiserum was replaced by serum from a rabbit injected with complete Freund adjuvant only in one circle and, in the other circle, from an uninjected rabbit (1:200 dilution). The reaction was photographed at 20 and 40 min after addition of substrate (a and b, respectively) and after completion (c). All intensities of reaction, from 1+ through 4+, can be seen, as well as initial precipitate formation (b: second circle from the left upper row). Well-formed precipitates are seen in b (uppermost left circle) and c. The figure demonstrates that the controls were consistently negative and that antibodies were detectable up to the highest dilution tested. It also shows agreement of readings before and after completion of the reaction. Thus the reaction pattern solidified by the precipitates in c reflects accurately the results of the assay and can be filed without further treatment as a permanent document.
different phenotypes by IF in antibody excess (Fig. 6a-d).

**DISCUSSION**

All 19 methanogen strains examined were immunogenic in rabbits, in agreement with our earlier studies in mice (unpublished data; Conway de Macario et al., Fed. Proc. 39:677, 1980). All elicited antibodies that reached high serum titers when administered after an immunization procedure that had previously been found efficient to raise antibodies to a purified *Escherichia coli* antigen (5). Anti-methanogen antibodies were demonstrable by immunofluorescence and immunoenzymatic techniques. The antisera were titrated and organized in a bank to serve as a source of material for preparation of antibody probes for immunological characterization of methanogens (6). Formalized methanogens can be stored for months without any appreciable decay in antigenicity insofar as they consistently give a 4+ immunofluorescence reaction under optimal conditions. The possibility that, under restrictive conditions, e.g., a high antiserum dilution, some antigenicity may become undetectable earlier, remains open for investigation. This point deserves further study since analysis of antigenic relatedness may require the use of both diluted probes and stored reference bacteria. In this connection, we might add that the generation of a set of stable antigens (bacteria) for long time storage could be a useful complement to our panel of antibody probes for identification of new isolates, resolution of complex ecosystems, and taxonomic studies.

The data obtained by the IF technique, which has a well established record in bacterial identification (11), were used as a basis for the development and standardization of other assays which were simpler, less demanding, and/or more amenable to quantification than IF. The slide immunoenzymatic assay depends upon subjective evaluation of the antigen-antibody reaction as is the case for IF, but is simpler than the latter. Moreover, the slide immunoenzymatic assay does not require expensive equipment and is performed more rapidly than IF. Reading the reaction of one full slide test (eight circles) takes less than 1 min, whereas examination of the same number of IF reactions, including focusing the microscope and finding an adequate number of microscopic fields, requires much longer times (at least 1 min per circle). This advantage of the slide test is particularly useful when many samples have to be screened in search of antibodies or cross-reactions. The slide test is invaluable for disclosing antibody-forming microcultures during the process of establishing hybridomas for the production of monoclonal antibodies. Screening for producer cultures can be done much faster and with better sensitivity by means of the slide immunoenzymatic assay than IF (Conway de Macario et al., Fed. Proc. 40:1594, 1981; R. J. Jovell, E. Conway de Macario, A. E. Alito, M. J. Wolin, and A. J. L. Macario, Fed. Proc. 40:1124, 1981).

The tube immunoenzymatic assay is particularly useful for quantification of antigenic relatedness independently from IF. The assay with glass test tubes was found to be more satisfactory than the enzyme-linked immunosorbent assay performed in plastic multiwell plates (22), on which our method is based. The tube assay gave no reaction with normal sera and the control fluids we tested, including sera from rabbits and mice inoculated with Freund complete adjuvant and culture supernatants (Conway de Macario et al., Fed. Proc. 40:1594, 1981; Jovell et al., Fed. Proc. 40:1124, 1981). Therefore, the results are unequivocal as well as highly reproducible. Most importantly, the tube assay provides abundant reaction mixture with which quantitative data can be obtained with standard spectrophotometric equipment.

Although the immunoenzymatic assays offer a series of advantages with respect to IF, the latter provides unique information on the morphology of methanogens. This is an invaluable bonus derived from the immunological staining of methanogens because of two important reasons:
FIG. 5. Examples of the morphological types as revealed by the IF reaction with homologous antiserum: (a) coccobacilli—*M. smithii* ALI; (b) rods—*M. bryantii* MoHG; (c) long rods—*M. hungatei* 3F1; and (d) packets—*M. barkeri* 227. Magnification, ×4,000.

FIG. 6. Growth of *M. formicicum* MF in a pressurized atmosphere of 50% H₂ and 50% CO₂ formed shorter phenotypes (a) than growth in 80% H₂ and 20% CO₂ (b). The same occurred with other methanogens, e.g., *M. bryantii* MoH (c and d). However, these phenotypes did not differ in their antigenicity insofar as their IF reaction in antibody excess was the same (compare a versus b and c versus d).
(i) the specificity of the staining, and (ii) the pleomorphism of methanogens. In this respect, we found that the morphological variations shown by various methanobacteria species, as they were cultured under different conditions, were not accompanied by detectable changes in antigenicity. Thus, the use of antibody probes along with immunological methods very likely will become an extremely useful way of distinguishing immunologically different but morphologically similar methanogens present in the same sample.

ACKNOWLEDGMENTS
We thank E. Currenti and R. J. Jovell for assistance.
This work was supported by North Atlantic Treaty Organization Research Grant No. 26.80, Department of Energy Grant DE-AC02-81ER10880, and Public Health Service Grant AI-12461 from the National Institutes of Health.

LITERATURE CITED