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Improved Agar Bottle Plate for Isolation of Methanogens or Other Anaerobes in a Defined Gas Atmosphere

MONIQUE HERMANN,† KENNETH M. NOLL, AND RALPH S. WOLFE*
Department of Microbiology, University of Illinois, Urbana, Illinois 61801

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A bottle plate for the cultivation of methanogens or other organisms in a defined pressurized-gas atmosphere was developed. The bottle provides the convenience of an agar streak plate, solves the problem of the water exudate from agar medium, and provides a convenient way of adding or sampling a defined gas atmosphere.

A modification of general procedures for the isolation of anaerobes in agar deeps was developed by Hungate decades ago (8, 9). The Hungate roll tube provides a more direct access to colonies. For the cultivation of hydrogen-oxidizing anaerobes, a gas atmosphere of hydrogen and carbon dioxide can be added to the center space. Streaking of roll tubes provides a convenient way of obtaining isolated colonies from a mixed inoculum and avoids dilution of the inoculum in melted agar (7). An agar bottle plate was used by Uffen and Wolfe (12) to provide strict anoxic conditions, this procedure being a modification of the Hungate technique. More recently, a similar procedure was used by Braun et al. for isolation of hydrogen-oxidizing acetogens (4). In roll tubes as well as in bottle plates, water that exudes from the agar medium during incubation collects, in contact with the agar, at the bottom of the vessel, allowing motile anaerobes to migrate in a film on the agar surface. In addition, the culture vessels must be handled with special care so that the exudate does not wash over the colonies on the agar surface.

The simplicity and convenience of the petri dish for the isolation of bacteria on an agar medium have never been surpassed; yet the use of the petri dish for the isolation of fastidious, nonsporeforming extreme anaerobes has severe limitations. It was not until the comparatively recent development of anaerobic chambers (1) that use of the petri dish for isolation of these organisms became practicable. Techniques for the cultivation of methanogens in an anaerobic chamber on petri dishes were first developed by Edwards and McBride (5), and later these procedures were modified for growth of methanogens on petri dishes in a pressurized atmosphere (2, 3, 10). The use of petri dishes for the cultivation of these organisms requires a considerable investment in equipment.

We describe in this communication an agar bottle plate that (i) provides the convenience of a streak plate for obtaining isolated colonies, (ii) solves the problem of the water exudate that may remain in contact with the agar, and (iii) provides a simple way of adding or sampling a defined gas atmosphere, which may be pressurized, if desired.

The bottle (Fig. 1) measures about 5.5 by 3 by 13.5 cm. The significant feature is the crease on one side that separates the agar chamber (the agar plate) from the chamber into which the water exudate drains. The vessel (catalog no. 2535 S 0020), complete with cap and stopper, is supplied by Bellco Glass, Inc., Vineland, N.J. The bottle has a black rubber stopper (no. 2049-11800) described previously (2, 3). Medium that contains 2% agar or 1% GELRITE (6) may be prepared anoxically by the Hungate procedure and dispensed into each bottle (10 ml per bottle) by appropriate use of gassing probes and a pipette (8, 9). Each black rubber stopper is lightly greased on the sides with silicone grease and is slipped into place as the gassing probe is withdrawn. The cap is then screwed in place. Alternatively, the vessel of anoxic medium may be sealed and transferred into an anaerobic chamber (1), where the medium may be easily

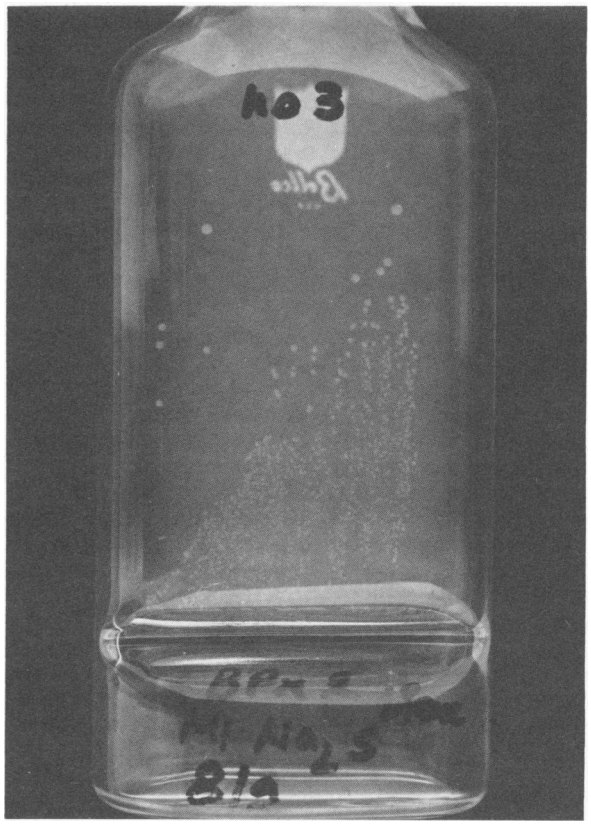

FIG. 1. Agar bottle plate showing isolated colonies in the upper chamber, the agar plate. The lower chamber collects water exuded from the agar medium. The agar chamber measures about 5.5 by 8 cm.

* Corresponding author.
† Present address: Institut Français du Pétrole, 92506 Rueil Malmaison, France.

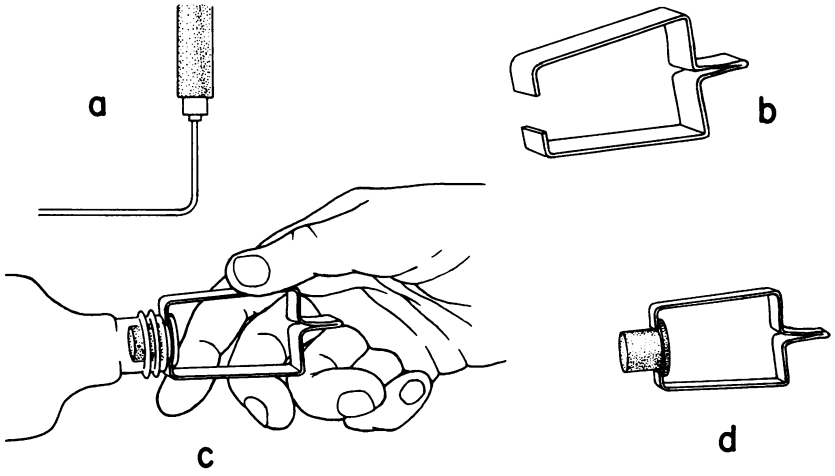

FIG. 2. Procedure and tool for removal of stopper from bottle plate. A sterile gassing probe (a) is positioned near the bottle so that the bottle opening may be placed quickly over it as the stopper is removed. A special tool (b) made of stainless steel is convenient for grasping the stopper (c) and for holding it on the bench top (d).

dispensed into bottles which are then stoppered and transferred out of the chamber.

The desired gas atmosphere is then added to each bottle by use of a gassing manifold and vacuum pump (2, 3); we recommend that 1 atm (1 atm = 101.29 kPa) of pressure be employed during autoclaving. Sterile anoxic solutions may be injected with a syringe into the bottle of sterile melted agar (cooled to about 60°C); for example, filter-sterilized

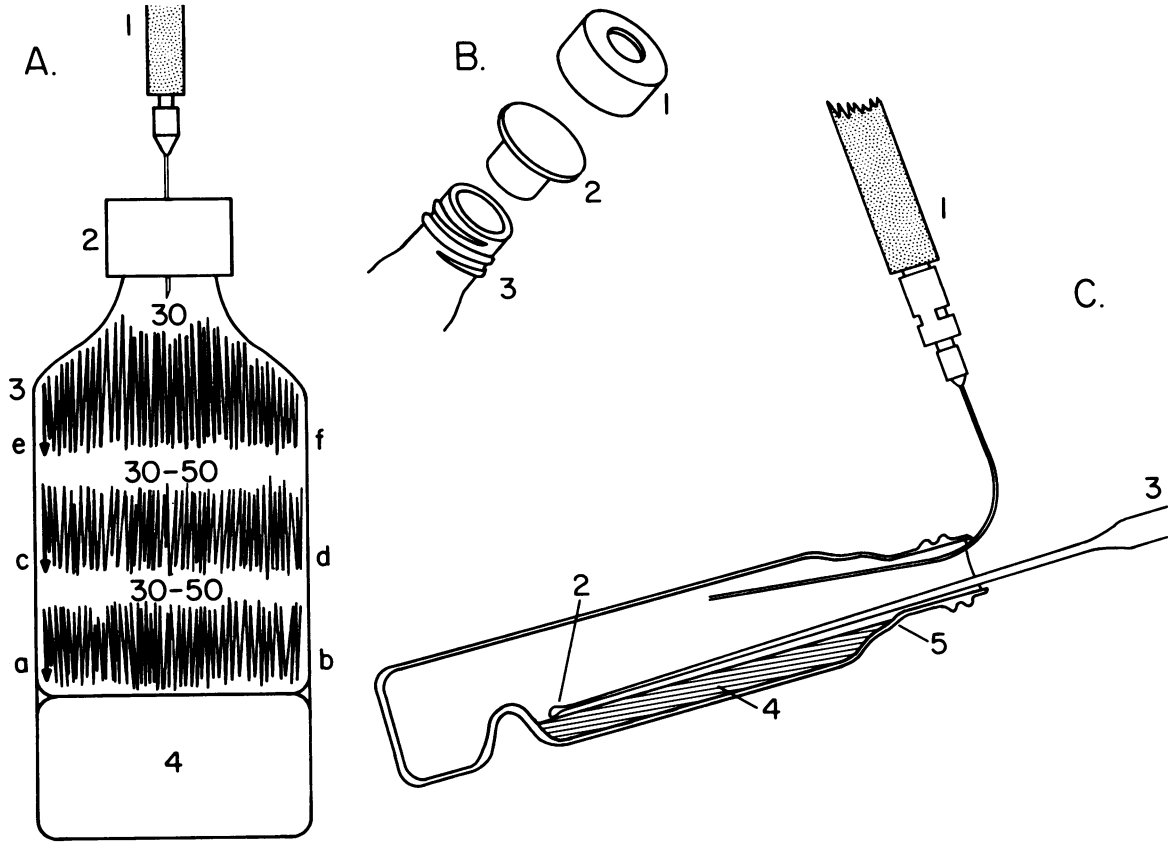

FIG. 3. Use of agar bottle plate. (A) The complete system with the atmosphere pressurized by use of a probe (A1) attached to a gassing manifold. The plastic cap (A2) holds the stopper in place. The agar plate area (A3) may be streaked as indicated (a to b, d to c, and e to f) with 30 to 50 cycles per section depending on the concentration of the inoculum. The chamber (A4) collects water exudate from the agar medium. (B) Plastic cap with center opening (B1) and rubber stopper (B2). (C) Streaking the agar plate. A sterile gassing probe (C1) is inserted. The melted end (C2) of a sterile Pasteur pipette (C3) is used to streak agar medium (C4) in the bottle plate (C5).

solutions of heat-sensitive substrates or a reducing agent such as dithionite may be injected at this stage. A sterile bottle of melted complete medium is inverted, so that the agar medium is collected in the capped end, and the bottle is gently placed on its side with the groove next to the bench top. After solidification of the agar, the bottle may be stored in an upright position. Poured plates may be stored for many weeks; storage in an anaerobic chamber increases the shelf life.

To streak a bottle plate, the rubber stopper is removed (Fig. 2). With a flamed gassing probe (Fig. 2a) positioned nearby, a simple tool (Fig. 2b) is used to grasp the lip of the rubber stopper and remove it (Fig. 2c). As the stopper is removed, the gassing probe is quickly inserted into the bottle. The tool serves as a convenient holder (Fig. 2d) for the stopper while colonies are picked or the bottle is streaked. The tool is fabricated from a strip of 21-gauge 304 stainless steel (12 by 160 mm).

In the streaking procedure (Fig. 3C), the gassing probe is held by a clamp attached to a ring stand, and the end of the bottle rests on the bench top. An inoculum is deposited from a pipette, syringe, loop or needle (Fig. 3A, position a). The small end of a sterile Pasteur pipette, melted in a flame, is used as a probe to streak the inoculum (4) (Fig. 3C). Use of a wire loop for streaking requires considerable skill to avoid tearing the agar surface. The bottle opening restricts freedom of lateral streaking movement, so a pattern of streaks parallel to the long axis of the bottle (Fig. 3A) is recommended. If a heavy inoculum is streaked 30 to 50 cycles over a 2-cm-wide path from positions a to b, d to c, and e to f (Fig. 3A), isolated colonies may be readily obtained. A second plate streaked from a much diluted inoculum is shown in Fig. 1. Before streaking, an inoculum may be conviently diluted by suspension in the water exudate in the lower chamber. Colonies may be picked with a platinum or stainless steel wire or a drawn, sterile-cotton-plugged Pasteur pipette attached to a mouth tube. The needle of a sterile 1-ml syringe is convenient for picking and transferring a second plate colony to liquid medium (3, 11). The syringe and pipette are flushed with the anoxic gas that exits through the neck of the bottle before use.

After the plate is streaked, the bottle is sealed, and the plastic cap (Fig. 3B1) is screwed in place. The gas used in the streaking procedure, usually N_2-CO_2 (80:20), may be replaced with H_2-CO_2 (80:20) or another gas mixture by means of a sterile gassing probe (Fig. 3A1) attached to a vacuum pump and gassing manifold (2, 3). For hydrogen-oxidizing anaerobes we use 2 atm of pressure in the bottle. The bottle is incubated in an upright position at the desired temperature.

Although we have used the bottle plate for isolation of methanogens and other strict anaerobes, we envision that the bottle should be an effective vessel for isolation of methanotrophs, aerobic hydrogen oxidizers, H_2S-oxidizing aerobes, anoxic photosynthetics, and microbes that may utilize a volatile compound placed in the bottom of the bottle.

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