Development of a Micromethod for Identification of Anaerobic Bacteria

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Received for publication 16 June 1975

A microprocedure was described for determining the carbohydrate fermentation patterns of 48 anaerobic bacteria at one time in microtiter plates. The cultures were transferred into agar-filled wells of microtiter plates with a replicator inside an anaerobic glove box. Fermentation was measured both with a colorimetric indicator and with a small pH electrode. The method was approximately 97% accurate. It would be most useful for laboratories that need to identify large numbers of anaerobes at one time.

Specific identification of anaerobic bacteria is based in large part on their fermentation patterns with a large range of carbohydrates. Determination of such reactions in individual tubes places a severe limit on both the number of organisms that can be examined and, just as important, the number of times analyses can be repeated on important organisms to ensure accuracy. Faced with both of these problems, we developed a method for performing such tests on 48 organisms at one time in microtiter plates. The use of microtiter plates and other compartmented plates for fermentation tests on aerobic bacteria has been reviewed recently by Fung and Hartman (3). We adapted these procedures for use with anaerobic bacteria and designed a replicator for transferring the cultures into the microtiter plates (7). Each plate contains a separate carbohydrate in an agar medium. Inoculation and incubation occur inside an anaerobic glove box. This paper describes the various factors considered during development of the method and the accuracy and reproducibility of the procedure.

MATERIALS AND METHODS

Replicator. The design and use of the replicator is described in an accompanying paper (7). The principle is that of a Steer's phage replicator (6) modified for stab inoculation of bacterial cultures into the agar-filled wells of microtiter-type plates.

Preparation of media. The base medium contained: 1% (wt/vol) Trypticase (BBL), 0.5% (wt/vol) yeast extract (Difco), 0.5% (wt/vol) NaCl, 0.25% (vol/vol) Tween 80 (Baker), and 1% (vol/vol) of a solution (4) of hemin and menadione to give 5 and 5 μg/ml, respectively, in the final medium. As a colorimetric pH indicator 5% (vol/vol) of a 0.2% (wt/vol) phenol red solution was added to all media, with the exception of the media used for esculin hydrolysis and gelatin digestion. The base medium was adjusted to pH 7.6 with 6 N NaOH and decanted into separate flasks for each substrate to be tested, 0.7% (wt/vol) agar (Difco) was added, and the flasks were autoclaved aerobically for 15 min at 121 C. The medium was then cooled to 70 to 80 C in a water bath, and a filter-sterilized solution of cysteine-hydrochloride was added to give a final concentration of 0.05% (wt/vol). All substrates with the exception of glyogen, starch, and esculin were filter sterilized as 5% (wt/vol) solutions and added aseptically to the medium to give a final 0.5% concentration. Glycogen, starch, and esculin could not easily be filter sterilized, so 5% (wt/vol) solutions were autoclaved aerobically (121 C for 15 min) and added while hot to the base medium. Gelatin (0.4%, wt/vol) was autoclaved with the basal medium, and a filter-sterilized glucose solution (5%, wt/vol) was added to the autoclaved gelatin medium to give a final 0.5% (wt/vol) concentration. The final pH of all media, after addition of cysteine and exposure to the atmosphere of the anaerobic chamber, was 6.9 to 7.1.

Carbohydrates tested. The following carbohydrates were tested: amygdalin, arabinose, cellobiose, erythritol, esculin, fructose, glucose, glycogen, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, starch, sucrose, trehalose, and xylene.

Dispensing of media. The media were dispensed aerobically while still hot (70 to 80 C) into the wells of sterile microtiter plates (Falcon, no. 3040) by the use of a 5-ml continuous pipetting syringe (Cornwall, Becton-Dickinson Co.) connected to an 8-prong dispenser (7). The syringe was set to fill one row of eight wells with 0.25 ml of media per well with each stroke of the plunger. Sterile plastic tops (Falcon, no. 3041) were used to protect the plates from contamination prior to and immediately after dispensing the media. Normally at least four plates of each type of medium were filled, and the syringe was then flushed completely several times with sterile boiling water and then with the next medium to be dispensed. After the plates had cooled, they were incubated overnight in an aerobic incubator at 37 C.
This was done both as a check for contamination and to dry the plates slightly. The plates were then placed in the anaerobic glove box and allowed to reduce for at least 2 days prior to use. For longer storage the plates were stored inside the glove box in plastic bags to prevent excess drying.

**Inocula.** Cultures to be tested were grown in rubber-stoppered tubes of prereduced media (4) inoculated under oxygen-free CO\textsubscript{2} on the bench top. These cultures were incubated at 37 C overnight and then placed in the anaerobic chamber. Unless stated otherwise, the cultures were grown on slants (10 ml) of prereduced sweet E (4) medium inoculated with one to 1.5 ml of a chopped meat (4) or E medium (4) broth culture of the organism. This yielded very highly turbid cell suspensions for use as inocula. For some experiments, chopped meat broth cultures (24 h) were used directly as inocula.

**Inoculation.** Inside the anaerobic glove box, the rubber-stoppered tubes containing the inocula were opened, and by the use of sterile Pasteur pipettes fitted with rubber bulbs the tubes in the sterile master plate of the replicator were filled almost to the top. Only 48 cultures were routinely tested at one time, and these were dispensed in a “checkerboard” pattern with 48 control wells. The replicator was then used to inoculate the plates; the prongs were charged with inocula between each plate.

**Sealing of wells.** To reduce cross-contamination of wells by motile bacteria and diffusion of volatile fatty acids from well to well, it was necessary to cover each plate with sterile plastic tape (nontoxic plate sealers, Cooke Engineering Co., Alexandria, Va.). The plate sealers were applied by laying the tape, with adhesive side up, on a firm rubber mat and pressing the inverted plate onto it. The plates were then rolled with a tape roller (Cooke Engineering Co.) to insure a firm seal on each well. To allow for the escape of gases produced during fermentation it was necessary to punch pin-point holes in the tape over each well with a tape perforator (Cooke Engineering Co.).

**Incubation.** The sealed plates were stacked in a 37 C incubator inside the anaerobic glove box. One of the plastic tops was placed on the top plate in each stack to reduce evaporation. Unless stated otherwise, incubation was for 3 days.

**Determination of results.** Fermentation of carbohydrate substrates was determined both by changes in the colorimetric pH indicator and by the actual pH of the medium as compared to inoculated basal media without added carbohydrate. To measure pH, a small pH electrode (Sargent no. S-30070-10) was inserted into the medium of each inoculated well. This electrode would stay upright in the well without support while the pH reading was recorded. It was not necessary to rinse the electrode between wells, since the agar in the well pushed any material carried over from the last well away from the tip. A pH value of 5.6 to 6.0 was considered as a weak fermentation and 5.5 and below was considered as acid.

Esculin hydrolysis was determined by observation of the black color produced by the addition of 1% ferric ammonium citrate to the agar wells. Digestion of gelatin was determined by the method of Frazier (2) by adding bichloride of mercury to the wells and observing a cleared zone around the stab.

**Anaerobic glove box.** A chamber similar to that described by Aranki and Freter (1) was used (Coy Manufacturing Co., Ann Arbor, Mich.). When media were introduced, the vacuum in the inlet port was allowed to drop to 20 inches (ca. 50 cm) on the gauge, and a series of three partial evacuations was used. The chamber contained an atmosphere of 5% CO\textsubscript{2}, 10% H\textsubscript{2}, and 85% N\textsubscript{2}.

**Tests of reducing agents.** For some experiments cysteine was replaced with dithioerythritol (0.01%), formaldehyde sulfoxylate (0.02%), or 2-mercaptoethanol (0.05%) in the culture media. For some experiments palladium chloride was incorporated in the media either as a purified powder or as a 5% acid (HCl) solution in concentrations ranging from 80 to 500 \(\mu\)g/ml.

**Bacterial strains.** Three sets of cultures were used. Group A consisted of 96 strains representing 52 species of anaerobes. These strains were representative of both common human fecal isolates (most common 32 species reported by Moore and Holdeman [5]) and clinical isolates (20 species). Group B was composed of cultures used for teaching purposes at the Virginia Polytechnic Institute (V.P.I.) Anaerobe Laboratory and consisted of one strain each of 69 species, the majority of which were clinical isolates. Group C consisted of a random assortment of 250 isolates sent to the V.P.I. Anaerobe Laboratory for confirmation of identification. The majority of these cultures were clinical isolates.

Tests on all cultures were performed by methods given in the V.P.I. Anaerobe Laboratory Manual (4) as well as with the replicator system. All such companion tests were under the direction of L. V. Holdeman and all identifications were done by either L. V. Holdeman or W. E. C. Moore.

**RESULTS AND DISCUSSION**

**Basal medium.** The composition of the basal medium for the replicator system was based on the following considerations. (i) It had to be nutritionally adequate for a wide spectrum of organisms; (ii) it had to have an oxidation-reduction (O-R) potential low enough to allow growth of most anaerobic bacteria; and (iii) it had to have a low buffering capacity. We started with the simple peptone-water medium routinely used for fermentation tests with enteric organisms and modified this as necessary to develop a suitable medium.

From preliminary trials it was obvious that a solid medium would be easier to work with, since liquid media could not be easily passed through the vacuum port of the glove box and spilled easily during manipulations. The choice of 0.7% agar was based on the requirement that the media had to be solid, yet soft enough for easy insertion of a pH electrode. Media with 0.7% agar also were easier to dispense than
Reducing agents. To grow many anaerobic bacteria it is necessary to use a medium with a low O-R potential. The best way to achieve this is to prepare the media completely anaerobically and add a reducing agent such as cysteine. For our purposes it was impractical to prepare the media anaerobically, since this would have necessitated dispensing the media inside the anaerobic chamber. We prepared the media aerobically and reduced the O-R potential of the media by exposure to the reducing atmosphere of the anaerobic chamber. We prepared media with or without cysteine (0.05%) and tested the effect of 0 to 4 days of exposure of the media to the glove box atmosphere. The number of the strains of group A that fermented glucose each day is given in Fig. 1. It was evident that both a preliminary period of reduction and the continued presence of the reducing agent were necessary to achieve fermentation with many strains. The optimum was 3 to 4 days of exposure of plates containing cysteine to the glove box atmosphere. The amount of cysteine required was examined in a similar experiment with plates reduced 3 days (Fig. 2). The highest amount of cysteine tested (0.05%) gave the most positive fermentations. Higher amounts of cysteine lowered the pH of the medium too much to be used and therefore were not tested. In similar trials we tested other reducing agents (dithioerythritol, formaldehyde sulfoxalate, and mercaptoethanol), but these were all slightly less effective than cysteine. Palladium chloride was also tested since it has been reported to be effective in reducing the O-R potential when incorporated into agar media (1). We tried this compound both as a powder and as an acid-solubilized liquid at concentrations ranging from 80 to 500 µg/ml. In the absence of cysteine, the compound was toxic and, when included with cysteine, we could not detect any improvement over cysteine alone. Perhaps with anaerobes more sensitive to O-R potential than those tested, palladium chloride might be a useful additive. However, in the presence of palladium chloride some strains of anaerobes turned the medium black. This interfered with reading the results of some tests.

Yeast extract. Many anaerobes require growth factors that can be supplied by incorporation of yeast extract in media. We tested this requirement in our system (Fig. 3). Yeast extract concentrations of between 0.125 and 0.5% resulted in an increase of 24% in the number of strains of group A that fermented glucose. For routine work we used 0.5% yeast extract.

Tween 80. The growth of some anaerobes is stimulated by oleic acid that can be supplied as Tween 80. We tested varying concentrations of Tween 80 (Fig. 4) and found that 13% of the strains of group A required this supplement for fermentation of glucose. We chose to use 0.0125% Tween 80 in our media. In our test only one isolate (a strain of Bacteroides melaninogenicus) was found to be inhibited by this concentration. Higher concentrations than 0.0125% did inhibit other strains.

Incubation time. The number of days of incubation required to achieve the maximum number of positive fermentation reactions was tested (Fig. 5). Within 24 h 88% of the cultures
of group A that could produce acid had done so. Incubation for longer than 3 days produced no further increase over the number of fermentations detected at 3 days. For routine use we measured fermentation after 3 days of incubation.

Inoculum. The source of inoculum did not appear to be important as long as young (12 to 24 h), actively growing cultures were used. We found that it was easier to grow the inocula in anaerobic tubes outside the glove box and introduce these tubes just before use. This resulted in a lower contamination rate than when cultures were transferred inside the glove box.

Contamination. Contamination of wells of the plates has been much less than 1% in our trials over the past 2 years with this system. Technologists must use care, however, and some experience is necessary to keep all manipulations aseptic. Contamination due to swarming of motile organisms was reduced greatly by the use of the plastic tape (nontoxic plate sealers). Very motile clostridia can still cause contamination if the plates are not well dried, and we do not routinely test such cultures.

Volatile fatty acids. When loose plastic tops were used instead of the plastic tape for sealing the wells during incubation, false-positive acid reactions sometimes resulted. This was due to production of large amounts of volatile fatty acids by some cultures. The fatty acids moved from well to well in the gaseous phase, and then dissolved in the media and lowered the pH of the adjacent wells. Bifidobacteria were particularly active in this regard. The plastic tape eliminated this problem. Pinpoint holes were punched in the tape over each well, allowing any gas produced to escape, but fatty acids could not diffuse into other wells at a rapid enough rate to change the pH. As a constant check on both contamination and diffusion of volatile fatty acids, we routinely inoculate only 48 of the 96 wells per plate in a "checkerboard" pattern. If uninoculated wells show either growth or acid reactions the organisms in that area of the plate are tested again.

Determination of results. For fermentation reactions, phenol red is included in the medium as a pH indicator. However, some anaerobes can lower the O-R potential sufficiently to completely eliminate the color of the indicator without lowering the pH. Although this is usually easy to differentiate from the yellow color of acid reactions, it can be confusing. Some organisms can also reduce the pH of the basal medium that does not contain added carbohydrate. Because of these problems, it is necessary to be
able to measure the pH of all wells that are not obviously red (i.e., not acid or reduced). This can easily be done with a small, combination pH electrode.

**Accuracy and reproducibility of the method.** Determination of the accuracy of a method for measuring fermentation reactions can be done in part by comparison to results obtained with an already accepted method. We compared our results with those obtained with the methods described in the V.P.I. Anaerobe Laboratory Manual, which are based on the use of prereduced media in rubber-stoppered tubes. This is the routine method used in our department, and the technologists performed these tests as part of their normal procedure.

Two different sets of cultures were examined. One set consisted of cultures used for teaching purposes (class cultures) and contained 69 species of anaerobic bacteria (group B). The second set was a random assortment of 250 isolates sent to the Anaerobe Laboratory for confirmation of identification (group C). The majority of these cultures were clinical isolates. With the 69 class cultures there was 96.3% overall agreement of individual tests between the two methods. There were 67 total disagreements in the 1,825 tests. In 43 of these disagreements, the replicator gave an acid reaction, and the tubes were negative. The reproducibility of the replicator method was determined by testing these same organisms two more times. The percentage of reproducibility in this test was 98.9%.

In tests conducted during the past year on the 250 random strains, there was an overall agreement of 96.6% of individual tests between the two methods. There were a total of 212 disagreements in the 6,250 tests, and in 117 of these the replicator indicated a positive reaction. The least agreement was with amygdalin (93.5%), fructose (92.6%), and ribose (92%). With fructose and ribose the disagreements were approximately equally distributed in regard to which method yielded positive reactions, but among the disagreements with amygdalin positive replicator results outnumbered positive tube results by 18 to 3. The reason for this result is unknown. Occasional organisms were found among these clinical isolates that appeared to ferment several sugars by one method and none by the other, but these were approximately equally distributed as to which method yielded positive reactions. Usually these organisms were found to yield variable results by either method.

We have used the replicator system on approximately 7,000 fecal isolates during the past 2 years, and the majority of these were identified based on the replicator data without the necessity of performing any tube tests. However, the replicator would not give reproducible reactions with some fecal isolates. Many of these organisms have complex growth requirements that were satisfied in the tube medium by addition of rumen fluid, but some appeared to require the lower O-R potential of the prereduced tube media. This was most common with the *Eubacterium rectale* groups (5) and *Fusobacterium prausnitzii*. Strains of *Eubacterium aerofaciens* frequently produced negative results for esculin, cellulose, and salicin with the replicator procedure but produced positive results in tube tests. It is important to remember that, just as with other methods, it is possible to get reproducible but inaccurate results with certain strains.

**Advantages and disadvantages.** We have found the method to be useful in our own laboratory and consider that it should be of use to other workers who deal with large numbers of cultures. However, it would not be useful for laboratories that only occasionally need to determine carbohydrate reactions or have small numbers of organisms to test at one time. The techniques are specialized and require experience; we have found that it is best to assign one technologist to the procedure instead of having several people use the equipment.

The primary advantage of the replicator method is its low cost per test. In our laboratory a single technologist uses this procedure to test approximately 200 cultures on 27 substrates per week. All media preparation, inoculation, and recording of data is performed by the same person, and some time is still available for other projects. The method thus allows analyses to be repeated several times on organisms of special interest and allows the usefulness of new tests to be analyzed rapidly on a wide variety of organisms.

**ACKNOWLEDGMENTS**

The help of L. V. Holdeman and W. E. C. Moore in supervising the companion tube tests and helping to interpret results is greatly appreciated. The technical assistance of Mike Weaver and Susan West on a portion of this work is gratefully acknowledged.

This work was supported by Public Health Service grant no. GM 14906 from the National Institute of General Medical Sciences and grant no. NO1-CP-33334 from the National Cancer Institute.

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