

3758

Microfermentation Series for Identification of Single Colonies of *Enterobacteriaceae*

C. N. HUHTANEN, J. NAGHSKI, AND E. S. DELLAMONICA

Microfermentation tests for members of the family *Enterobacteriaceae* were devised by using agar solutions in disposable, multi-welled, plastic trays. The tests could be made directly from isolated colonies picked from agar plates and represented a considerable saving in time, labor, and materials over the conventional methods. Tests were formulated for determining carbohydrate fermentations, citrate utilization, motility, amino acid decarboxylation, and production of H₂S, indole, urease, and acetyl-methyl-carbinol.

Recent literature, replete with references to rapid methods for bacterial identification, indicates the great interest and need for these "short-cut" methods. A compilation of recent rapid methods was made by Hartman (15) in 1968; however, many advances in the field have occurred since. The necessity for more rapid identification methods is apparent for the *Enterobacteriaceae*, especially *Salmonella*, from foods and feeds. The rapid movement of animal feed ingredients from production at the rendering plants to final blending at the feed mill, for instance, demands quick methods of identification of contaminating microorganisms.

Some of the recent work on rapid methods involves the use of suspensions of bacterial cells. Borchardt (3), Matsen and Sherris (18), Gandelman (12), and Grunberg et al. (14) have described and evaluated methods by using heavy cell suspensions in contact with paper-impregnated substrates. Such reagent-impregnated strips are available commercially (Patho Tek, General Diagnostics Division, Warner-Chilcott Laboratories, Morris Plains, N.J.). Paper discs impregnated with carbohydrates were placed on agar plates heavily seeded with cells in rapid fermentation tests devised by Sanders et al. (22) and Schafer et al. (23). Another approach, not requiring heavy inocula, was described by Grunberg et al. (14) and was compared to the paper strip method. This method, also commercially available (Enterotube, Hoffman-La Roche, Inc., Nutley, N.J.), consists of eight substrates in a multi-compartmented tube; a built-in needle is touched to a colony and then drawn succes-

sively through the compartments. Fluorescent-antibody methods for *Salmonella* have been described by a number of workers (Ellis and Harrington [9], Georgala and Boothroyd [13], Insalata et al. [17], and Reamer et al. [21]). These show considerable promise, especially for *Salmonella* identification. Methods for identifying bacteria by gas chromatography have been described by Henis et al. (16) and O'Brien (20). The identification of bacteria from single colonies, together with analysis by computer, was described by Corlett et al. (5). The method was fast and provided identification of 10 different genera based on a scheme involving sensitivity to antibiotics and growth characteristics on different agars. Another recent method, the Analytab system (Analytab, Inc., N.Y.) was evaluated by Washington et al. (24); it showed considerable promise for identifying *Enterobacteriaceae* from single colonies. Bergquist and Searcy (2) developed a multi-agar column method for determining several biochemical characteristics in a single tube by using inoculum from one colony. Microtiter plates (multi-welled plastic trays) were used by several workers for differing purposes. Davies et al. (6) adapted them for carbohydrate fermentations by *Neisseria meningitidis*; Fung and Miller (11) studied the carbohydrate fermentations of 25 species of bacteria; Fuccillo et al. (10) and Catalano et al. (4) used them for growing mammalian cells in tissue culture. The method described in this report is based on the use of colonies obtained directly from primary isolation plates using similar multi-welled plastic trays.

MATERIALS AND METHODS

Cultures. The cultures used were *Escherichia coli* ATCC 26, *Enterobacter aerogenes* ATCC 13048, *Citrobacter freundii* ATCC 8090, *Providencia* ATCC 12013, *Arizona arizonae* ATCC 12323, *Klebsiella pneumoniae* ATCC 132, *Pseudomonas aeruginosa* ATCC 8709, *Proteus* sp. (this was originally classified as *Proteus vulgaris* ATCC 4669, but the culture was indole negative and was most likely *P. mirabilis*), and laboratory isolates of *Salmonella alachua*, *S. maderia*, *S. tennessee*, *S. anatum*, *S. muenchen*, *S. reading*, *S. gaminara*, *S. newington*, *S. meleagridis*, *S. montevideo*, and *Hafnia* sp. The cultures were streaked onto MacConkey agar, and isolated colonies were used as the inocula.

Meat-and-bone meal samples. The meat-and-bone meal samples were some that had been shown to contain salmonellae by using conventional techniques. These samples had been incubated in a tetrathionate enrichment medium and streaked on brilliant green agar plates. Two colonies had been picked from each plate and inoculated into triple sugar iron and lysine iron agar (Difco) slants and confirmed as salmonellae by serotyping. Colonies from the same plates, held several days in a refrigerator, were used in the agar microfermentation series.

Multi-welled trays. The plastic multi-welled trays were obtained from the Linbro Chemical Co., New Haven, Conn. Each tray had 96 compartments or wells arranged in 8 rows of 12 wells each. Each well had a capacity of approximately 0.3 ml; we used 0.2 ml of agar per well. Plastic covers (no. 53) were used with the trays. The trays and covers (not autoclavable) were sterilized by dipping in 95% alcohol and drying at 37 C.

Microfermentation media. The carbohydrate media consisted of commercial phenol red or bromocresol purple broth bases (Difco) with 1.5% added agar. Separately autoclaved 10% carbohydrate solutions were added for a final 1% concentration. The H₂S, indole, and motility tests were made on sulfide-indole-motility (SIM, Difco) medium. Simmons citrate agar (formulated from individual ingredients) was used for determining citrate utilization. The Voges-Proskauer test was made on methyl red-Voges Proskauer (MR-VP, Difco) medium with 1.5% added agar. The medium for the amino acid decarboxylase tests consisted of: nutrient broth, 0.8 g; amino acid (L-lysine dihydrochloride, L-arginine monohydrochloride, or L-ornithine monohydrochloride), 0.5 g; agar, 1.5 g; 0.1 M KH₂PO₄, 3.0 ml; 0.1 M Na₂HPO₄·7H₂O, 1.6 ml; 0.04% phenol red (H₂O solution), 3.0 ml; and H₂O to 100 ml. The medium for the urease test consisted of: peptone, 0.1 g; NaCl, 0.5 g; KH₂PO₄, 0.2 g; agar, 0.2 g; 0.04% phenol red, 3.0 ml; and H₂O to 90 ml. Five percent carboxy-methyl-cellulose (Avicel, American Viscose Corp.) was made up as a dispersant for the inoculum. All media and the carboxy-methyl-cellulose solution were autoclaved at 15 psi for 10 min. After cooling to 50 to 55 C, 10 ml of a Seitz-filtered 1% solution of urea was added to the 90 ml of urea agar.

Conventional biochemical tests. The media used

for conventional tests were: for carbohydrate fermentations, phenol red broth base with 0.5% separately autoclaved carbohydrates; for H₂S, indole, and motility, SIM medium (BBL); for Voges-Proskauer, MR-VP medium; for lysine decarboxylase, lysine iron agar; for citrate, Simmons citrate agar (formulated from ingredients); and for urease, urea broth (Difco). Kovac's reagent was used for the indole test and α -naphthol and KOH for the Voges-Proskauer test. The carbohydrate tests were incubated for 7 days at 37 C before being classed as negative, whereas the SIM and urea media were incubated for 2 days at 30 and 37 C, respectively. The designation of the reactions in the various tests were: +, positive, \pm , weak positive or doubtful, and -, absence of a characteristic. Reduction of dye in the carbohydrate media with no apparent acid reaction was indicated by R.

Inoculations. Isolated colonies were picked from streaked plates with a short needle (45 mm) having a 2-mm diameter loop on the end. The colonial growth was mixed with carboxy-methyl-cellulose solution in the first well. The cultures were inoculated horizontally into the rows of wells while the media were added vertically to the columns of wells. The loop was not flamed until all inoculations involving one culture were made. Inoculations were made from this well into the center of each succeeding well in the row, returning each time to the carboxy-methyl-cellulose inoculum. Clear plastic covers, furnished by the Limbro Chem. Co., were placed on the plates which were then incubated at 37 C with observations being made at 18, 24, and 48 hr. All tests were fully developed at 18 hr unless otherwise noted.

Observation of reactions. Color changes were observed by placing the trays on a fluorescent light box (Glow-box, 1st R Co., Cheltenham, Pa.). The indole test was performed by using one drop of Kovac's reagent delivered from a Pasteur pipette. The Voges-Proskauer reaction was observed after the addition of one drop of 5% α -naphthol in absolute alcohol and one drop of 40% KOH.

RESULTS

Development of the microfermentation series. Urease reaction. The first tests on urea breakdown, using two strains of *Proteus*, were made by using urea agar base (Difco) containing 0.1% peptone, 0.1% glucose, 0.5% NaCl, 0.2% monopotassium phosphate, 2% urea, and 0.012 g of phenol red/liter with 1.5% agar. This medium worked well when *Proteus* was tested alone, but neighboring wells of the trays became alkaline due to production of excess NH₃ from the urea. The amount of urea was successively decreased in the medium until, at a concentration of 0.1%, there was a barely perceptible pink tinge in neighboring wells. This concentration was used in subsequent tests.

Further studies were made on the influence of

glucose levels on urease reactions by several other organisms. The results (Table 1) showed a strong urease reaction with *Citrobacter freundii* ATCC 8090 and *Klebsiella pneumoniae* ATCC 132 at pH 6.7, particularly when the medium contained 0.1% glucose as in the original Difco formulation. The reaction was fainter with 0.2% glucose and absent when no glucose was added. There were no effects of glucose or pH with *Salmonella alachua*, the *Hafnia* sp., or *Escherichia coli* ATCC 26; all were urease negative. The two strains of *Proteus* showed the best reactions without glucose either at pH 6.0 or at 6.7; with 0.1% glucose, reactions were strong at pH 6.7 but weak at 6.0; and with 0.2% glucose, reactions were faint. The final medium used for our studies was made without the glucose and at a pH of 6.7; this readily detected the urease of *Proteus*. For detecting the weaker urease activity of *Klebsiella* or *Citrobacter*, the use of 0.1% glucose at pH 6.7 would be recommended. The combination urease-phenylalanine medium of Ederer et al. (7) with added agar might also be of value in this test.

Amino acid decarboxylation reactions.

Preliminary tests for lysine decarboxylase were made by using a simple medium (Difco nutrient broth) with varying levels of phosphate buffer and 0.5% lysine HCl. When the final concentration of phosphate buffer was 0.0125 M or higher, no alkalinity was observed. A concentration of 0.006 M gave some color, and 0.003 M was better. A final concentration of 0.0046 M was used in subsequent experiments; this gave a good color reaction without being too heavily buffered.

One of the problems encountered in early media formulations was with *Citrobacter*, which produced a false positive lysine decarboxylase reaction (Table 2). Apparently, this was a result of ammonia production from the peptone, since addition of 0.1% or 0.2% glucose caused the apparent lysine decarboxylation reaction to disappear. Two strains of *Salmonella* and *Hafnia* did not show any diminution of alkalinity when glucose was added or at the two initial pH values tested (6.0 and 6.6). The medium finally chosen was one with

TABLE 1. Effect of glucose and pH on urease test^a

Culture	Intensity of alkaline reaction					
	No glucose		Glucose 0.1%		Glucose 0.2%	
	pH ^b 6.0	pH 6.7	pH 6.0	pH 6.7	pH 6.0	pH 6.7
<i>Salmonella alachua</i>	-	-	-	-	-	-
<i>Citrobacter freundii</i> ATCC 8090	-	±	±	+++	±	+
<i>Proteus</i> sp. (1)	+++	+++	+	+++	±	+
<i>Hafnia</i> sp.	-	±	-	-	-	-
<i>Proteus</i> sp. (2)	+++	+++	+	++	-	+
<i>Escherichia coli</i> ATCC 26	-	-	-	-	-	-
<i>Klebsiella pneumoniae</i> ATCC 132	-	±	+	++	±	+

^a Basal medium: peptone, 0.1 g; agar, 1.5 g; NaCl, 0.5 g; KH₂PO₄, 0.2 g; 0.04% phenol red, 3 ml; and H₂O to 90 ml. Before use, 10 ml of Seitz-filtered 1% urea solution was added.

^b Initial pH values (all columns).

TABLE 2. Effect of glucose and pH on lysine decarboxylase test^a

Culture	Development of alkaline reaction					
	No glucose		Glucose 0.1%		Glucose 0.2%	
	pH ^b 6.0	pH 6.6	pH 6.0	pH 6.6	pH 6.0	pH 6.6
<i>Citrobacter freundii</i> ATCC 8090	+++	+++	-	±	-	-
<i>Salmonella alachua</i>	+++	+++	++	+++	++	+++
<i>S. madelia</i>	+++	+++	++	+++	++	++
<i>Hafnia</i> sp.	+++	+++	+++	+++	++	+++
<i>Escherichia coli</i> ATCC 26	+	++	-	±	-	-
<i>Proteus</i> sp.	±	±	-	-	-	-
<i>Providencia</i> ATCC 12013	-	±	-	-	-	-

^a Basal medium: nutrient broth, 0.8 g; agar, 1.5 g; 0.1 M Na₂HPO₄·7H₂O, 4.6 ml; 0.04% phenol red, 3 ml; lysine hydrochloride, 0.5 g; and H₂O to 100 ml. pH adjusted with 1 N HCl.

^b Initial pH values (all columns).

0.1% glucose and with an initial pH of 6.6. The other two amino acids, ornithine and arginine, were successfully tested for decarboxylation in the same basal medium. The reactions of several of the *Enterobacteriaceae* are shown in Table 3. *E. coli*, *Enterobacter aerogenes* ATCC 13048, *A. arizonae* ATCC 12323, *S. alachua*, and *Hafnia* decarboxylated all three amino acids. *Citrobacter* decarboxylated arginine and ornithine and was negative on lysine and arginine. *Providencia* sp. ATCC 12013 was negative on all three amino acids.

Tests for motility and production of acetyl-methyl-carbinol, indole, and H₂S. Difco SIM medium was used for the indole, motility, and H₂S production tests. The inoculations were made directly in the centers of the wells. Motile cultures grew in a diffuse zone throughout the well and were clearly differentiated from the nonmotile growth of *K. pneumoniae* (Table 4). Hydrogen sulfide production was noted as a black precipitate and was heaviest along the line of inoculation. Indole production was clearly evident with *E. coli* after the addition of one drop of Kovac's reagent (Table 4). A clear, positive test was obtained with *Enterobacter aerogenes* and *K. pneumoniae* with the Voges-Proskauer test.

Citrate utilization. The test for citrate utilization using the Simmons citrate formulation proved more troublesome than any of the other tests. Table 4 shows the reactions of the selected group of enteric organisms. Rapid (18 hr) positive reactions were evident with *Enterobacter aerogenes*, *S. alachua*, and *K. pneumoniae*, whereas negative reactions, even after

48 hr of incubation, were obtained with *Providencia* and *Hafnia*. *E. coli* was negative at 18 hr and weakly positive after 48 hr. *Citrobacter*, *Arizona*, and *Proteus* sp. were negative at 18 hr, weakly positive at 24 hr, and strongly positive at 48 hr. Attempts were made to make these reactions more rapid by decreasing the concentration of K₂HPO₄, or by adding increments of NH₄Cl as an extra source of nitrogen. Decreasing the K₂HPO₄ to 0.06 or 0.01% (normal concentration is 0.1%) did not give clearer color changes. The addition of increments of NH₄Cl to 0.1% final concentration also did not improve the reactions. Adjustment of the pH to 6.45, the point where there is only a green color in the medium, from the normal 6.8 (a blue-green color) also was without effect on the rapidity or clarity of the citrate reactions.

Carbohydrate fermentations. Table 5 shows some carbohydrate reactions in the microfermentation test using phenol red broth base. Most of the reactions were clear-cut, especially when viewed over the fluorescent light. A notable exception was *Enterobacter aerogenes*, when showed clearly positive reactions with raffinose, salicin, sucrose, and xylose, but doubtful reactions in most of the other carbohydrates. Similar reactions were seen in other tests with purple broth base.

Effect of serial inoculation of wells. Tests were made on the efficiency of the microfermentation series when a colony was picked with a needle and inoculated serially into the agar wells without the preliminary preparation of an

TABLE 3. Amino acid decarboxylase reactions in microfermentation series^a

Culture	Amino acid		
	Arginine	Lysine	Ornithine
<i>Escherichia coli</i> ATCC 26	+	+	+
<i>Enterobacter aerogenes</i> ATCC 13048	+	+	+
<i>Citrobacter freundii</i> ATCC 8090	+	-	+
<i>Arizona</i> ATCC 12323	+	+	+
<i>Proteus</i> sp.	-	-	±
<i>Providencia</i> ATCC 12013	-	-	-
<i>Salmonella alachua</i>	+	+	+
<i>Hafnia</i> sp.	+	+	+

^a Symbols: +, Positive reaction (red color); ±, doubtful positive reaction; -, negative reaction. Test medium: glucose, 0.1 g; nutrient broth, 0.8 g; 0.04% phenol red, 3.0 ml; 0.1 M Na₂HPO₄·7H₂O, 4.6 ml; amino acid, 0.5 g; agar, 1.5 g; water to 100 ml, pH 6.6.

TABLE 4. Indole, H₂S, and acetyl-methyl-carbinol production, citrate utilization, and motility^a

Culture	H ₂ S	Indole	Motility	Voges-Proskauer	Citrate
<i>Escherichia coli</i> ATCC 26	-	+	+	-	+ ^b
<i>Enterobacter aerogenes</i> ATCC 13048	-	-	+	+	+
<i>Citrobacter freundii</i> ATCC 8090	+	-	+	-	+ ^c
<i>Arizona</i> ATCC 12323	+	-	+	-	+ ^c
<i>Proteus</i> sp.	+	-	+	-	+ ^c
<i>Providencia</i> ATCC 12013	±	-	+	-	-
<i>Salmonella alachua</i>	+	-	+	-	+
<i>Hafnia</i> sp.	-	-	+	-	-
<i>Klebsiella pneumoniae</i> ATCC 132	-	-	-	+	+

^a Incubation for 18 hr at 37 C unless otherwise noted.

^b Weakly positive after 48 hr.

^c Negative after 18 hr; weakly positive after 24 hr; strongly positive after 48 hr.

inoculum in the carboxy-methyl-cellulose. In this test, the needle was not flamed after the colony was picked. In one test using *Arizona*, 72 successive wells were inoculated before any aberrant reaction was noted. In another test, 53 wells were inoculated successfully. A third test with a *Salmonella* strain was made by inoculating four complete microfermentation series (in order: lysine, dulcitol, lactose, maltose, salicin, sorbitol, sucrose, xylose, Simmons indole motility, MR-VP, and urea) for a total of 44 stabs with no aberrant reactions noted.

Comparison of the microseries with conventional tests. Carbohydrate fermentations. A comparison of the reactions of several *Enterobacteriaceae* using the agar microfermentation technique (microtest, MT) with those obtained in conventional tests (CT) is shown in Table 6. Acid production from arabinose, raffinose, and sucrose was the same with all cultures except *Enterobacter aerogenes*, which showed only slight acid production from arabinose in the MT but had a normal acid reaction in the CT. The dulcitol reactions were also similar except for *E. coli*, which produced acid from the CT but not from the MT. *S. madelia* produced only slight acid from dulcitol in the MT and none from the CT (the indicator dye was reduced); the other salmonellae were the same in the two tests. Glucose, maltose, and mannitol fermentations were similar in the two tests, although again *Enterobacter aerogenes* produced more acid in the CT. In general, lactose fermentations gave similar results although *Enterobacter aerogenes* and *K. pneumoniae* reduced the dye in the CT while producing slight acid in the MT. Rhamnose was fermented by *Enterobacter aerogenes* in the CT but not in the MT; the other

organisms showed similar reactions in both tests. Salicin reactions were similar except that *E. coli* showed a dye reduction in the CT and a negative reaction in the MT. Sorbitol tended to be fermented more vigorously in the CT by *E. coli* and *Enterobacter aerogenes*; the other organisms showed similar reactions with both techniques. These two organisms also showed acid production from the CT but none in the MT. *Hafnia* tended to produce more acid in the xylose CT whereas *K. pneumoniae* produced less. It was noted that the acid reactions in the MT tended to revert rather quickly to alkaline when the plates were kept at either room or refrigeration temperature. This was most apparent with xylose and dulcitol.

Comparison of other tests. *Providencia* showed a negative indole reaction in the MT used in these comparisons whereas the CT was positive; in other MT, however, the indole reaction was positive. *Pseudomonas aeruginosa* showed a positive indole reaction in the MT and a negative reaction in the CT. Motility was the same in both tests as were the Voges-Proskauer, citrate, H₂S, and urease reactions. Lysine decarboxylase tests also correlated very well except for *Pseudomonas aeruginosa*, which was negative in the CT and questionable in the MT.

Influence of inoculation media on carbohydrate fermentations. The possible effect of brilliant green dye in the growth medium on subsequent fermentation reactions was investigated. Overnight cultures from nutrient agar slants were streaked onto brilliant green agar, and the reactions of isolated colonies were compared with the reactions of nutrient agar slants incubated overnight (about 18 hr). The reactions of salmonellae on all sugars except

TABLE 5. Carbohydrate fermentation reactions^a

Culture	Arab- inose	Dul- citol	Glu- cose	Lac- tose	Mal- tose	Man- nitol	Raf- finose	Rham- nose	Sal- icin	Sor- bitol	Su- crose	Xy- lose
<i>Escherichia coli</i> ATCC 26	+	-	+	+	+	+	-	+	-	±	-	-
<i>Enterobacter aerogenes</i> ATCC 13048	±	-	±	±	±	±	+	-	+	±	+	+
<i>Citrobacter freundii</i> ATCC 8090	+	-	+	+	+	+	-	+	-	+	±	-
<i>Arizona</i> ATCC 12323	+	-	+	-	+	+	-	+	-	+	-	-
<i>Proteus</i> sp.	-	-	+	-	-	-	-	-	-	-	-	-
<i>Providencia</i> ATCC 12013	-	-	+	-	-	-	-	-	-	-	-	-
<i>Salmonella alachua</i>	+	+	+	-	+	+	-	+	-	+	-	+
<i>Hafnia</i> sp.	+	-	+	-	+	+	-	+	-	-	-	±
<i>Klebsiella pneumoniae</i> ATCC 132	+	-	+	±	+	+	+	+	+	+	+	+

^a Basal medium: phenol red broth base (Difco) with 1.5% agar; carbohydrates added to 1%, separately autoclaved.

TABLE 6. Comparison of reactions in conventional and microfermentation series

Culture	Reactions in microfermentation series ^a																	No. of reactions differing out of 19	
	Arabinose	Dulcitol	Glucose	Lactose	Maltose	Manitol	Raffinose	Rhamnose	Sorbitol	Sucrose	Xylose	H ₂ S	Indole	Motility	Voges-Proskauer	Lysine decarboxylase	Urea		Citrate
<i>Escherichia coli</i> ATCC 26	+	-(+)	+	+	+	+	+	+	±(+)	-	-(+)	-	+	-	-	+	-	±(-)	5
<i>Enterobacter aerogenes</i> ATCC 13048	±(+)	-	±(+)	±(R)	±(+)	±(+)	-	-(+)	±(+)	+	+	-	-	+	+	+	-	+	7
<i>Citrobacter freundii</i> ATCC 8090	+	-	+	+	+	+	+	+	+	±	-	+	+	+	-	-	-	±	0
<i>Providencia</i> ATCC 12013	-	-	+	-	-	-	-	-	-	-	-	-	-(+) ^b	-	-	-	-	-	1
<i>Arizona arizonae</i> ATCC 12323	+	-	+	-	+	+	+	+	+	-	+	+	-	+	-	+	-	+ ^c	0
<i>Salmonella alachua</i>	+	+	+	-	+	+	+	+	+	-	+	+	-	+	-	+	-	+	0
<i>Hafnia</i> sp.	+	+	+	-	+	+	+	+	-	-	±(+)	+	-	+	+	+	-	-	1
<i>S. eimsbuettel</i>	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	-	+	0
<i>S. madela</i>	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	-	+	1
<i>S. tennessee</i>	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	-	+	0
<i>Klebsiella pneumoniae</i> ATCC 132	+	+	+	±(R)	+	+	+	+	+	+	±(±)	-	-	-	+	+	-	+	2
<i>Pseudomonas aeruginosa</i> ATCC 8709	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	±(-)	-	+ ^c	2
<i>Proteus</i> sp.	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	0
No. of reactions differing out of 13	1	2	1	2	1	1	0	1	2	0	3	0	2	0	0	1	0	1	19 ^d

^a Parentheses are reactions of conventional tests when there were differences. R, Reduction of indicator; acid production obscured, if present.

^b Indole was positive in microseries in another test.

^c Negative after 24 hr, positive after 48 hr.

^d Overall differing reactions were 8% of the total (247).

dulcitol were the same. *S. newington* grown on nutrient agar fermented dulcitol, but brilliant green agar colonies did not. The reverse was true with *S. meleagridis* and *S. montevideo*.

Further studies of the comparison of reactions of cultures grown on triple sugar iron or nutrient agar slants were made. The only difference noted with *E. coli* was with trehalose which was not fermented by the nutrient agar cultures but was with the triple sugar iron. *Enterobacter aerogenes* showed the most differences of any of the organisms tested. In general, this organism produced more acid when grown in nutrient agar. *Citrobacter* grown on triple sugar iron failed to ferment sorbitol or sucrose but these sugars were fermented by the nutrient agar cultures. *Arizona*, *Proteus*, and *Providencia* showed no differences. *S. alachua* fermented dulcitol only when grown on the nutrient agar. The *Hafnia* showed only a slight difference in mannitol fermentation.

Comparison of 7.5- and 24-hr incubation times. The small size of the fermentation media in the wells and the relatively large inoculum prompted an experiment to determine whether the reactions of this group of bacteria could be observed within the course of a normal working day. Trays were inoculated in the morning and observed after 7.5 hr of incubation. Duplicate trays were observed at 24 hr.

The comparison of these two incubation periods is given in Table 7. The four salmonellae fermented dulcitol at 24 hr but not at 7.5 hr. Most of the other sugars were fermented more vigorously at 24 hr. Motility and H₂S were fully apparent at the earlier time. The indole reaction of *E. coli* was evident at 7.5 hr, but the production of acetyl-methyl-carbinol by *K. pneumoniae* and *Hafnia* was not evident until 24 hr.

Usefulness for naturally contaminated material. A study of the efficiency of the micromethod for detecting salmonellae in naturally contaminated meat-and-bone meal is shown in Table 8. Three rendering plants (numbered 5, 27, and 30) were sampled. Ten samples were taken from each plant. The meat-and-bone meal samples were incubated in tetrathionate broth (30 g of sample to 100 ml of broth) for 24 hr and streaked onto brilliant green agar plates. Colonies showing a negative lactose fermentation reaction were selected and inoculated into the microfermentation series as shown in Table 8. Mannitol, sorbitol, and xylose were included in the tests for purposes of determining the reactions of freshly isolated cultures; the results were not used in the presumptive analysis of the organisms. Presumptive identification of salmonellae was based on a positive reaction for lysine

TABLE 7. Effect of 7.5- versus 24-hr incubation period on microfermentation reactions^a

Culture	Arab- inose	Dul- citol	Galac- tose	Glu- cose	Lac- tose	Mal- tose	Man- nitol	Raf- finose	Rham- nose	Sor- bitol	Su- crose	Xy- lose	In- dole	Voges- Pros- kauer
<i>Escherichia coli</i> ATCC 26	+	-	+	+	+	+	±(+)	-	±(+)	- (±)	-	-	+	-
<i>Pseudomonas</i> <i>aeruginosa</i> ATCC 8709	-	-	-	-	-	-	-	k-	-	-	-	-	-	-
<i>Klebsiella</i> <i>pneumoniae</i> ATCC 132	+	-	+	+	- (±)	+	+	+	+	±(+)	+	- (+)	-	- (+)
<i>Salmonella</i> <i>madelia</i>	+	- (±)	+	+	-	±(+)	+	-	+	±(+)	-	+	-	-
<i>S. tennessee</i>	+	- (+)	+	+	-	±(+)	+	-	+	±(+)	-	- (+)	-	-
<i>Enterobacter</i> <i>aerogenes</i> ATCC 13048	± (±)	-	± (±)	± (±)	- (±)	± (±)	± (±)	- (±)	± (-)	±	+	- (+)	-	-
<i>Hafnia</i> sp.	+	-	+	+	-	- (+)	- (+)	-	- (+)	-	-	± (±)	-	± (+)
<i>S. eimsbuettel</i>	+	- (+)	+	+	-	+	+	-	+	± (+)	-	+	-	-
<i>Arizona</i> ATCC 12323	+	-	+	+	-	- (+)	- (+)	-	- (+)	- (+)	-	± (-)	-	-
<i>S. alachua</i>	+	- (+)	+	+	-	± (+)	± (+)	-	- (+)	± (+)	-	- (+)	-	-
<i>Citrobacter</i> <i>freundii</i> ATCC 8090	+	-	+	+	± (+)	± (+)	+	-	± (+)	± (+)	± (±)	± (-)	-	-
<i>Providencia</i> ATCC 12013	-	-	-	-	-	-	-	-	-	-	-	± (-)	- (±)	-
<i>Proteus</i> sp.	-	-	+	±	-	-	-	-	-	-	-	-	-	-

^a Reactions in parentheses are changes at 24 hr from 7.5 hr. Salicin fermentation reactions, H₂S productions, and motility omitted from this table since there were no differences between 7.5- or 24-hr incubation periods.

decarboxylase and motility with negative reactions for lactose, sucrose, indole, and urea. The cultures were, in addition, inoculated into triple sugar iron and lysine iron agar slants. Cultures having salmonella-like or arizona-like (negative dulcitol) reactions in these were serotyped.

Sample 5-10 showed five positive colonies out of five picked; three of these were classified tentatively as salmonellae-arizonae. Confirmation showed all five to be salmonellae including the dulcitol-negative cultures (*S. montevideo* and *S. eimsbuettel*). Sample 27-2 showed two possible salmonellae; these were confirmed as *S. senftenberg* and *S. cerro*. Sample 27-6 showed one possible salmonella which failed to

ferment dulcitol. This was identified as *S. senftenberg*. *S. cerro* was also isolated from samples 27-8 and 30-8. Two cultures from samples 27-8 and 30-7, which failed to produce H₂S, were tentatively identified as salmonellae but did not agglutinate with any of the polyvalent somatic antisera. The four urease-positive cultures classed as *Proteus* were negative on dulcitol, mannitol, and sorbitol, thus differentiating them from *Klebsiella*. They were, however, positive for acetyl-methyl-carbinol and were, most likely, *P. mirabilis*. The salmonellae were, in general, positive on mannitol, sorbitol, and xylose; the single exception was a negative sorbitol reaction by one of the *S. cerro* isolates. The salmonellae are generally regarded as being

TABLE 8. Identification of salmonellae from meat-and-bone meal by using the microfermentation series^a

Meat-and-bone meal sample	Colony	Lysine decarboxylase	Dulcitol	Lactose	Mannitol	Sorbitol	Sucrose	H ₂ S	Motility	Voges-Proskauer	Urea	Presumptive identification	Final identification		
5-10	a	+	+	-	+	+	-	+	+	-	-	Salmonella	Group C ₁ <i>S. montevideo</i>		
	b,c	+	-	-	+	+	-	+	+	-	-	Salmonella-Arizona			
	d	+	-	-	+	+	-	+	+	-	-	Salmonella-Arizona	<i>S. eimsbuettel</i>		
	e	+	+	-	+	+	-	+	+	-	-	Salmonella	<i>S. oranienberg</i>		
	27-2	a	+	+	-	+	+	-	-	+	-	-	Salmonella	<i>S. senftenberg</i>	
	b	+	+	-	+	+	-	+	+	-	-	Salmonella	<i>S. cerro</i>		
	c,d,e,f	-	-	-	+	-	-	-	+	-	-				
27-6	a	+	+	±	-	±	+	-	+	+	-		<i>S. senftenberg</i>		
	b	+	-	-	+	+	-	-	+	+	-	Salmonella?			
27-8	a,b	-	+	-	+	-	-	-	+	-	-		<i>S. cerro</i>		
	c	+	-	-	+	-	-	+	+	-	-	Salmonella			
	d	±	-	-	-	-	-	+	+	+	+	Salmonella?		<i>Proteus</i>	
	e	+	-	-	+	-	+	+	+	+	-	Salmonella?		Negative ^b	
	f	-	+	-	+	+	-	-	+	+	-	-			
	g	+	+	-	+	+	-	+	+	-	-	-		Salmonella	<i>S. cerro</i>
															<i>Proteus</i>
30-5	a,b,c	-	-	-	-	-	-	+	+	+	+		<i>Proteus</i>		
	d,e,f,g	-	+	-	+	-	-	-	-	-	-				
30-7	a,b	+	-	-	-	-	-	-	-	+	-		Salmonella-Arizona		
	c	+	-	-	+	-	-	-	+	-	-				
	d	+	-	-	-	-	-	-	+	+	-				
30-8	a	-	+	-	+	+	-	-	+	-	-		Salmonella		
	b	+	+	-	+	+	-	+	+	-	-				
	c	+	+	-	+	+	-	+	±	-	-				
30-9	a	-	-	-	+	+	-	-	+	-	-				
	b,c,d	-	+	-	+	+	-	-	+	-	-				

^a All cultures were indole negative, 27-2 colonies c,d,e,f and 27-8 colony f were xylose negative, and the others all were xylose positive.

^b Triple sugar iron reaction after MacConkey agar purification was A/A.

able to ferment mannitol and sorbitol (8).

DISCUSSION

Recommended microfermentation methods. Several of the tests were performed by using commercially available media, supplemented where needed with 1.5% agar. These were tests for acid production from carbohydrates, H_2S and indole production, motility, Voges-Proskauer, and citrate utilization. These were generally equivalent to the conventional tests although little credence should be attached to the citrate test. The best medium for urease production was patterned after urea agar base, except that glucose was omitted and 0.1 instead of 2% urea was added. The best medium for the amino acid decarboxylase tests was similar to decarboxylase medium base (Difco), except that 1.5% agar was added, and the medium was lightly buffered with phosphate (3 ml of 0.1 M KH_2PO_4 and 1.6 ml of 0.1 M $Na_2HPO_4 \cdot 7H_2O$ to 100 ml medium). Phenol red (0.0012%) was also used instead of bromcresol purple. We would recommend an overnight (18 to 24 hr) incubation period. Although a carboxymethyl-cellulose dispersing material for the inoculum was used in most of our studies, we believe this to be unnecessary. Reliable results were obtained by serial inoculation of the wells with a single needle pick of a colony.

Advantages of the method. The advantages of this microfermentation technique are in its flexibility, rapidity, and parsimonious use of materials and space. The method is flexible in that an analyst can choose whichever media he wishes to work with, depending on his own preference and area of interest. Although designed particularly for salmonella identification, the method can be used as well for other genera, excluding perhaps the obligate anaerobes, although an overlay of paraffin might preserve a reducing potential, thereby allowing these organisms also to be studied. The method is rapid, e.g., an entire disposable tray with 96 wells divided into 8 rows with 11 different media in each row, as in these studies, can be inoculated with 8 different colonies in a matter of minutes. The amount of agar and reagents required is very small, meaning that less material and equipment need to be handled during preparation, autoclaving, and incubation. The trays are disposable, eliminating the tedium of glassware washing.

Perhaps the biggest advantage of a method such as this is its usefulness for single colony identification without recourse to preliminary culturing in a selective or nonselective medium.

Salmonella presumptive colonies from streaks on brilliant green agar plates, for instance, are generally inoculated into a triple sugar iron medium, and, depending on the reactions in this medium, would then be inoculated into other media for biochemical testing or would be serotyped. In the microfermentation test, the colony would go directly to the media for biochemical testing.

There are pitfalls in the use of isolated colonies of bacteria growing on a highly selective medium such as salmonellae on brilliant green agar plates. Some colonies might contain organisms that are only temporarily inhibited and, when the colony is transferred to a less selective or nonselective medium, the formerly inhibited organisms might gain predominance and obscure the reactions of the organisms in question. The more transfers made, the greater is this possibility. The microfermentation method described here obviates this possible source of trouble to a considerable extent, since there is only one transfer involved into the nonselective media.

Overall drawbacks to the microfermentation series. Most of the problems of this microfermentation series seemed to be of a technical nature. Considerable condensation occurred on the underside of the plastic lid whenever changes in temperature occurred especially on removing the plates from the incubator to room temperature. This condensation made it difficult to read the reactions without removing the covers. The problem was alleviated somewhat if, following the incubation period, the covers were displaced until the plates had equilibrated to room temperature. Also, the agar in the wells had a tendency to dry out rapidly due to the small volumes of agar and the loose-fitting covers. Wrapping the plates in a clear plastic film (Saran Wrap, Dow Chemical Co.) helped prevent this dehydration. A more tightly fitting lid could perhaps be devised which would help prevent the moisture loss. Other solutions to the problem might be the use of overlays of wax, agar, or mineral oil. May (19) reported considerable success with oxyethylene docosanol in preventive dehydration of agar in aerosol samplers. Fung and Miller (11) used an Amojell overlay (1 part Amojell, American Oil Co., and 1 part mineral oil) for trapping gas in their miniature fermentation system. The use of such overlays deserves further study, particularly if inoculation can be made through the overlaying material.

Another disadvantage was the downward concavity of the lids, which caused the under-

side to come into contact with the medium when the wells contained more than 0.2 ml each. A better cover would solve this problem; in our experiments we used rubber bands placed around the long axis of the covers to remove the downward bulge. Considerable pipetting expertise was needed to place the 0.2-ml amounts of agar solutions in wells, although, with practice, we found this method to become quite rapid and efficient. Automatic pipettors such as the Cornwall syringe (Becton-Dickinson), Aliquantor (Hamilton Co.) and Digi-Pet (Manostat Corp.) are available from laboratory supply houses and might find utility in pipetting these solutions. We tried the Cornwall syringe (1-ml capacity), which worked well with water solutions, but it would not satisfactorily pipette the 0.2-ml portions of the agar solutions used in these experiments. The difficulties involved in cleaning and sterilizing these syringes when changing from one medium to another prompted us to forego extensive studies in this area. Where large numbers of plates are being prepared, it is possible that such devices would allow a more satisfactory pipetting procedure.

We sterilized our plates with a 95% alcohol dip; Davies et al. (5) used ultraviolet irradiation. Presterilized plates are also available (Cooke Engineering Co., Alexandria, Va.; Linbro Chemical Co., New Haven, Conn.; Canalso, Inc., Rockville, Md.).

The microfermentation tests were incapable of determining the gas production of these organisms, a useful characteristic for differentiating some species. The Amojell overlay of Fung and Miller (11) might be helpful in this determination.

LITERATURE CITED

1. Abrahamsson, K., G. Patterson, and H. Riemann. 1968. Detection of *Salmonella* by a single-culture technique. *Appl. Microbiol.* **16**:1695-1698.
2. Bergquist, L. M., and R. L. Searcy. 1964. Laboratory suggestion: multi-agar columns for identifying single colonies of enteric organisms. *Amer. J. Clin. Pathol.* **42**:298-299.
3. Borchardt, K. A. 1968. Simplified method for identification of enteric and other gram-negative bacteria using reagent-impregnated strips. *Amer. J. Clin. Pathol.* **49**:748-750.
4. Catalano, L. W., Jr., D. A. Fuccillo, and J. L. Sever. 1969. Piggy-back microtransfer technique. *Appl. Microbiol.* **18**:1094-1095.
5. Corlett, D. A., Jr., J. S. Lee, and R. O. Sinnhuber. 1965. Application of replica plating and computer analysis for rapid identification of bacteria in some foods. I. Identification scheme. *Appl. Microbiol.* **13**:808-817.
6. Davies, J. A., J. R. Mitzel, and W. E. Beam, Jr. 1971. Carbohydrate fermentation patterns of *Neisseria meningitidis* determined by a microtiter method. *Appl. Microbiol.* **21**:1072-1074.
7. Ederer, G. M., J. H. Chu, and D. J. Blazevic. 1971. Rapid test for urease and phenylalanine deaminase production. *Appl. Microbiol.* **21**:545.
8. Edwards, P. R., and W. H. Ewing. 1962. Identification of *Enterobacteriaceae*, 2nd ed. Burgess Publishing Co., Minneapolis.
9. Ellis, E. M., and R. Harrington, Jr. 1969. A direct fluorescent antibody test for *Salmonella*. *Arch. Environ. Health* **19**:876-881.
10. Fuccillo, D. A., L. W. Catalano, Jr., F. L. Moder, D. A. Debus, and J. L. Sever. 1969. Minicultures of mammalian cells in a new plastic plate. *Appl. Microbiol.* **17**:619-622.
11. Fung, D. Y. C., and R. D. Miller. 1970. Rapid procedure for the detection of acid and gas production by bacterial cultures. *Appl. Microbiol.* **20**:527-528.
12. Gandelman, A. L. 1966. The use of reagent impregnated paper strips as an aid in the identification of certain gram negative organisms. *Amer. J. Med. Technol.* **32**:85-87.
13. Georgala, D. L., and M. Boothroyd. 1964. A rapid immunofluorescence technique for detecting salmonellae in raw meat. *J. Hyg.* **62**:319-327.
14. Grunberg, E., E. Titsworth, G. Beskid, R. Cleeland, Jr., and W. F. Delorenzo. 1969. Efficiency of a multitest system (Enterotube) for rapid identification of *Enterobacteriaceae*. *Appl. Microbiol.* **18**:207-213.
15. Hartman, P. A. 1968. Miniaturized microbiological methods. *In* Advances in applied microbiology, suppl. 1. Academic Press Inc., New York.
16. Henis, Y., J. R. Gould, and M. Alexander. 1966. Detection and identification of bacteria by gas chromatography. *Appl. Microbiol.* **14**:513-524.
17. Insalata, N. F., S. J. Schulte, and J. H. Berman. 1967. Immunofluorescence technique for detection of salmonellae in various foods. *Appl. Microbiol.* **15**:1145-1149.
18. Matsen, J. M., and J. C. Sherris. 1969. Comparative study of the efficacy of seven paper-reagent strips and conventional biochemical tests in identifying gram-negative organisms. *Appl. Microbiol.* **18**:452-457.
19. May, K. R. 1969. Prolongation of microbiological air sampling by a monolayer on agar gel. *Appl. Microbiol.* **18**:513-514.
20. O'Brien, R. T. 1967. Differentiation of bacteria by gas chromatographic analysis of products of glucose catabolism. *Food Technol.* **21**:1130-1132.
21. Reamer, R. H., R. E. Hargrove, and F. E. McDonough. 1969. Increased sensitivity of immunofluorescent assay for *Salmonella* in nonfat dry milk. *Appl. Microbiol.* **18**:328-331.
22. Sanders, A. C., J. E. Faber, Jr., and T. M. Cook. 1957. A rapid method for the characterization of enteric pathogen using paper discs. *Appl. Microbiol.* **5**:36-40.
23. Schafer, W. J., R. E. Anderson, R. A. Morck, and W. E. Cassidy. 1968. Use of reagent tablets for rapid biochemical identification of salmonellae and other enteric bacteria. *Appl. Microbiol.* **16**:1629-1630.
24. Washington, J. A., II, P. K. W. Yu, and W. J. Martin. 1971. Evaluation of accuracy of multitest micromethod system for identification of *Enterobacteriaceae*. *Appl. Microbiol.* **22**:267-269.