JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1976, p. 291-301
Vol. 3, No. 3

Printed in U.S.A.

Modification of the Minitek Miniaturized Differentiation System for Characterization of Anaerobic Bacteria

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Received for publication 21 October 1975

The Minitek Miniaturized System (BBL) was modified for characterization of anaerobic bacteria. The modified system and the conventional Center for Disease Control method were used to test a variety of anaerobic bacteria, and results were compared. Tests performed by both techniques were indole and H_2S production, esculin hydrolysis, nitrate reduction, and fermentation of glucose, mannitol, lactose, sucrose, maltose, salicin, glycerol, xylose, arabinose, mannose, rhamnose, and trehalose. The manufacturer's recommended procedure for the Minitek system was modified by using a new suspension medium (Lombard-Dowell broth) and an inoculum equivalent to the density of a McFarland no. 5 nephelometer standard. The Minitek results, recorded after 48 h, agreed satisfactorily with the conventional test results, usually recorded after 5 to 7 days of incubation. In the examination of 80 strains representing 22 different species or subspecies of anaerobic bacteria, with 16 biochemical tests performed in triplicate, 93.8% of the Minitek test results agreed with those of the corresponding conventional tests. Only tests for indole, H_2S, and nitrate reduction gave less than 90% agreement. It was concluded that the modified Minitek system is a suitable substitute for the more expensive and time-consuming conventional procedure for determining carbohydrate fermentation and esculin hydrolysis by anaerobes. This system, when used in conjunction with other tests, can effectively aid in the definitive identification of commonly isolated anaerobes.

In 1973 we described our work with a micromethod system (Analytab Products, Inc., New York, N.Y.) for the performance of biochemical tests commonly used in the identification of anaerobic bacteria (17). The reactions obtained with the micromethod tests were compared with those obtained with analogous conventional tests used by the Center for Disease Control (CDC) Anaerobe Section for identification of anaerobes. In addition to those tests performed with both systems for comparison, other differential tests not available as micromethod tests, including gas-liquid chromatography, were found to be necessary supplementary tests for proper species identification of the organisms. In the study of 104 cultures, 91% of the total tests performed with the two systems were in agreement. Only 5 of the 17 differential tests compared gave less than 90% agreement. From this study, it was concluded that substituting rapid, economical, microbiochemical techniques for some of the more expensive, time-consuming conventional tests for identification of anaerobes is quite feasible. Moore et al. (11) subsequently reported that the API system maintained a satisfactory performance level in the characterization of anaerobes in their laboratory. Schreckenberger and Blazevic (13) have stated that certain PathoTec test strips used for the rapid characterization of anaerobic bacteria yielded encouraging results.

Recently BBL (Cockeysville, Md.) marketed the Minitek Miniaturized Microorganism Differentiation System for characterization of bacteria by observation of their effect upon various chemical substrates. The system consists of paper disks impregnated with appropriate substrates that are dispensed into the wells of a special Minitek plastic plate. A cell suspension of the test organism is inoculated into each of the wells with a special pipetting device used to dispense a premeasured volume of inoculum (Fig. 1). The plates are incubated under increased humidity for an appropriate time period, and then the reactions are read and recorded.

Other investigators have reported that the Minitek system is reliable for accurate characterization of the *Enterobacteriaceae* (7, 9). This report describes certain modifications of the Minitek system that were found to be necessary in the characterization of anaerobes and the results we obtained from testing a variety of anaerobic bacteria with the modified system.

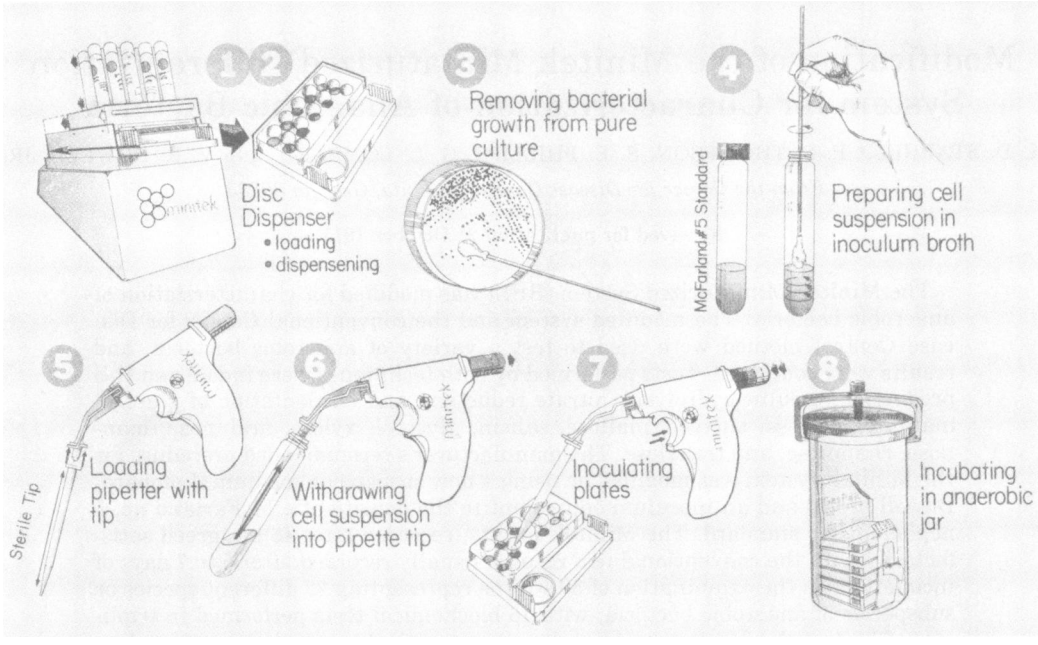

FIG. 1. *Schematic representation of the modified Minitek procedure for characterization of anaerobic bacteria.*

MATERIALS AND METHODS

Substrates tested. The biochemical tests performed by both the Minitek and the conventional systems were production of indole, urease, and H_2S; hydrolysis of esculin; reduction of nitrate; and the fermentation of glucose, mannitol, lactose, sucrose, maltose, salicin, glycerol, xylose, arabinose, mannose, rhamnose, and trehalose. Levulose fermentation was tested by the micromethod only.

Bacteria used. Initially, cultures referred to the CDC Anaerobe Section for identification were used as they were received to evalulate the Minitek system by the manufacturer's recommended procedure. During the remainder of the study, lyophilized cultures from the CDC stock culture collection were used. Among these were previously characterized clinical isolates as well as cultures derived from strains from the American Type Culture Collection, National Collection of Type Cultures, and Virginia Polytechnic Institute Anaerobe Laboratory.

Procedure. Cultures of clinical isolates that were referred to the CDC for identification were received in various fluid and solid transport media. Upon arrival in the laboratory, the specimens, handled as coded unknowns, were subcultured on the bench top in ambient atmosphere to freshly poured Trypticase soy-yeast extract blood agar medium supplemented with hemin-vitamin K1 (BA) described previously (6). After the plates were streaked to obtain isolated colonies, they were held under anaerobic conditions as described by Martin (10) in a gas mixture of 85% N_2, 10% H_2, and 5% CO_2. As the holding jars became full, the plates were transferred to an anaerobic glove box similar to that described by Aranki et al. (1) and were incubated for 48 to 72 h (or until growth was apparent). After colonies were visible, the plates were removed from the glove box, and the cultures were examined for purity with a dissecting microscope. One to three colonies of each pure culture were then transferred to a tube of thioglycolate medium, BBL 0135C, for subsequent inoculation of conventional biochemical media. Immediately after inoculation of the thioglycolate medium, four to six colonies from the same plate were suspended in 1.0 ml of the Minitek inoculum broth (MIB) supplied by the manufacturer in screw-capped vials. The appropriate disks were dispensed into wells, and each well was inoculated with approximately 0.05 ml of the cell suspension with the Minitek pipetting device. After a sterile mineral oil overlay (approximately 0.1 ml) was added to the H_2S and urea disks, the plates were quickly placed under anaerobic conditions in GasPak jars containing disposable GasPak generators (H_2 + CO_2) and new or reheated (2 h in a dry-heat oven at 160 C) catalyst pellets. After 48 h of incubation the Minitek plates were removed from the jars, the color changes of the disks were compared with the color reaction standards supplied by the manufacturer, and results were recorded. If the disks were colorless as a result of reduction of the indicator by the culture, 1 or 2 drops of an aqueous 0.025% solution of phenol red was added to each well to allow the reactions to be read. After the glucose reaction was recorded, reduction of nitrate was tested by adding 1 or 2 drops of nitrate A reagent (sulfanilic-acetic acid) and then 1 or 2 drops of nitrate B reagent (dimethyl-α-naphthylamine) to the dextrose-nitrate disk. Two to four drops of Kovac reagent was added to the H_2S-indole disk to

test for the presence of indole. The conventional biochemical tests were performed as described by Dowell and Hawkins (6); reactions were recorded after 48 h and after 5 to 7 days.

In addition to the tests performed by the two methods, we tested all cultures for Gram stain reaction, microscopic morphology, colony characteristics, hemolysis of rabbit blood, decomposition of hydrogen peroxide, motility, reactions in iron milk, hydrolysis of gelatin, and metabolic products produced in glucose-peptone-yeast extract broth as detected by gas-liquid chromatography. Suspected clostridium cultures were also tested for lecithinase and lipase activity on egg yolk agar and for mouse toxicity. Gram-negative nonsporeforming rods were tested for growth in the presence of 20% bile and for sensitivity to penicillin (2-U disk), and all cultures were tested for aerotolerance by surface streaking a nonreduced BA plate and incubating it in a candle jar. Growth in the candle jar was compared with that obtained anaerobically on the same medium.

The remainder of the study consisted of a two-stage evaluation of various modifications of the manufacturer's recommended procedure.

Stage 1: Preliminary evaluation of suspending media and inoculum size. Five well-characterized reference strains were used to test the effects of varying the inoculum size and the effects of using different suspension media. In addition, variability among multiple disks of the same substrate that were inoculated simultaneously and differences in individual interpretation of color reactions were analyzed. Lyophilized cultures of the test organisms were reconstituted and checked for purity on BA incubated aerobically and anaerobically. After a tube of thioglycolate medium was inoculated from a single colony for use in subsequent conventional testing, bacterial suspensions were prepared with sterile polyester fiber swabs in three different media: (i) MIB; (ii) thioglycolate broth without dextrose or indicator (Difco 0432-D1); and (iii) Lombard-Dowell broth (LDB). The LDB contained the following ingredients: Trypticase, 0.50%; L-tryptophan, 0.02%; yeast extract, 0.50%; sodium chloride, 0.25%; sodium sulfite, 0.01%; L-cystine, 0.04%; hemin, 0.001%; vitamin K1, 0.001%; and agar, 0.07%. The pH was adjusted to 7.4 ± 0.1 before autoclaving. Care was taken to avoid excessive aeration when suspensions were prepared. Two cell suspensions were prepared with each of the three media. The turbidity of one was adjusted to that of a McFarland no. 1 and that of the other to a McFarland no. 5 nephelometer standard. Each of the six different cell suspensions prepared with each strain was used to inoculate three complete sets of Minitek biochemical substrates (each set of disks in a separate Minitek plate). After the plates were incubated in a GasPak jar for 48 h, the reactions were read by four individuals. Only yellow was considered to be a positive reaction for carbohydrate fermentation, and all variations of orange-yellow were recorded as negative (as suggested by the manufacturer during the study). The results obtained with the Minitek tests for carbohydrate fermentation and esculin hydrolysis were then compared with the final results of the conventional tests.

Stage 2: Comparison of Minitek and Lombard-Dowell Suspension Media. Minitek tests were performed with LDB and MIB inocula, and results were compared. Eighty strains of anaerobic bacteria were used in the study. These included 20 reference strains and 60 clinical isolates, which represented a total of 22 species (see Table 5). All cultures were reconstituted from the lyophilized state, checked for purity, and then handled as coded unknowns. The LDB was stored under N_2 for no longer than 6 weeks before it was used. All cell suspensions were adjusted to the density of a McFarland no. 5 nephelometer standard. As in stage 1, the micromethod disks were tested in triplicate. A new lot of H_2S-indole disks was used in this stage of the study. The conventional tests were performed in the usual manner. After 45 to 50 h of incubation, the Minitek plates (an equal number in each of the jars) were removed from the GasPak jars and assigned random code numbers from a table of random integers; then they were arranged in numerical order to preclude the possibility of bias that might have existed if the reader had recorded triplicate reactions simultaneously. One investigator read each of the reactions.

RESULTS

The results obtained with the Minitek procedures recommended initially by the manufacturer and the results of the CDC conventional tests are shown in Table 1. Fifty strains representing 30 species of anaerobes were used to compare the two systems. Agreement between the two systems for 17 biochemical tests ranged from 48.6% to 98.0%; overall agreement was 79.5%. The ratio between positive results obtained with the micromethod only and positive

TABLE 1. *Observed agreement between Minitek and CDC conventional systems for characterization of anaerobic bacteria*

Substrate or test	No. of strains tested	Conventional − Minitek + (%)	Conventional + Minitek − (%)	Tests in agreement (%)
Urease	49	2.0	0	98.0
Arabinose .	50	2.0	6.0	92.0
Nitrate	50	0	12.0	88.0
Rhamnose .	50	2.0	12.0	86.0
Lactose	50	6.0	12.0	82.0
Esculin	50	0	18.0	82.0
Glycerol ...	50	2.0	18.0	80.0
Mannose ...	50	6.0	14.0	80.0
Trehalose ..	50	6.0	14.0	80.0
Glucose	50	2.0	20.0	78.0
Mannitol ..	50	6.0	16.0	78.0
Salicin	50	8.0	14.0	78.0
Sucrose	50	8.0	14.0	78.0
Xylose	50	4.0	22.0	74.0
Indole	35	11.4	17.1	71.4
Maltose	50	8.0	26.0	66.0
H_2S	35	40.0	11.4	48.6
Total tests .	819	6.0	14.5	79.5

results by the conventional method only was 1:2.5, although both types of discrepancies were frequent. Only the micromethod results with one carbohydrate disk (arabinose) and with the urease disk were in better than 90% agreement with conventional results.

Stage 1. An analysis of variance was performed to assess the effects of (i) varying the cell density of the inoculum, (ii) varying the suspension broth, and (iii) the individual interpretation of color reactions upon the reliability of the micromethod tests. Multiple readers did not differ significantly in their interpretation of positive and negative color reactions. Therefore, the data from four readers for three disks with each biochemical test at a particular cell density in each suspension broth were pooled to obtain a mean of 12 observations. These values were used for comparing the test medium with the standard medium. Overall, the results obtained with the denser inocula (cell density corresponding to a McFarland no. 5) were better than were results obtained with the less dense inocula (cell density corresponding to a McFarland no. 1). Both thioglycolate broth without carbohydrate and LDB inocula produced better results than MIB inocula, independent of the cell density used. In general, increasing the cell density from McFarland no. 1 to McFarland no. 5 resulted in a relative reduction of positive reactions with the conventional tests only and an increase in positive reactions with the micromethod tests only. The agreement between micromethod and conventional tests was best with LDB inocula at a McFarland no. 5 density and poorest with MIB inocula at a McFarland no. 1 density.

Stage 2. Table 2 shows results obtained with 80 test strains at a McFarland no. 5 cell density in a comparative study of MIB and LDB. An overall agreement of 85.2% with the micromethod and analogous conventional tests was obtained with the MIB inocula, whereas the tests with LDB inocula resulted in 93.8% agreement. In every instance but one (nitrate reduction), the percentage of agreement was better with the LDB inocula. When carbohydrate fermentation was considered separately, the percentage of agreement did not change appreciably for MIB inocula, but exceeded 96% for LDB inocula. Results with 12 of the 16 substrates tested (including 9 of the 12 carbohydrates) agreed with conventional test results less than 90% of the time when MIB inocula were used; in contrast, with LDB inocula only 3 of the 16 substrates tested agreed less than 90% of the time with the conventional system, and none of the carbohydrates were among these. In fact, only 2 of the 12 carbohydrates tested with LDB inocula showed less than 95% agreement—glucose (94.2%) and glycerol (92.1%). Whereas use of MIB inocula resulted in a large majority of negative reactions by the micromethod only, use of LDB inocula resulted in a slight majority of positive results by the micromethod only. H_2S and indole were the only two substrates

TABLE 2. *Agreement of results obtained with Minitek and CDC conventional systems with two types of suspension media used for testing 80 strains of anaerobic bacteria*[a]

Test	No. tested	Conventional − Minitek + (%)		Conventional + Minitek − (%)		Tests in agreement (%)	
		MIB	LDB	MIB	LDB	MIB	LDB
Lactose	240	0	0	12.5	0	87.5	100.0
Arabinose	240	0.8	0.8	0.4	0	98.8	99.2
Sucrose	240	0	2.1	10.4	0	89.6	97.9
Rhamnose	240	0	1.3	6.2	0.8	93.8	97.9
Trehalose	240	0	1.2	12.1	1.7	87.9	97.1
Mannitol	240	0	3.3	14.2	0.4	85.8	96.3
Maltose	240	1.2	0.8	19.6	2.9	79.2	96.3
Xylose	240	0.8	4.2	5.0	0	94.2	95.8
Salicin	240	0	0	19.2	5.0	80.8	95.0
Mannose	240	0.4	2.1	18.8	2.9	80.8	95.0
Esculin	240	0	4.6	14.2	0.8	85.8	94.6
Glucose	240	0	0	37.5	5.8	62.5	94.2
Glycerol	240	0	3.7	10.8	4.2	89.2	92.1
Nitrate	240	0.4	1.7	6.3	8.7	93.3	89.6
Indole	240	7.1	6.7	4.1	4.1	88.8	89.6
H_2S	240	26.7	21.2	9.1	7.5	64.2	71.3
Total	3,840	2.3	3.4	12.5	2.8	85.2	93.8
Total excluding H_2S	3,600	0.7	2.2	12.8	2.5	86.5	95.3
Carbohydrate fermentation only	2,880	0.3	1.6	13.9	2.0	85.8	96.4

[a] Each test was performed with triplicate disks inoculated simultaneously with the same cell suspension.

tested which, in both types of suspension media, gave results that agreed less than 90% of the time with conventional results. Each of these tests gave a majority of positive reactions by the micromethod only, independent of the type of suspension medium used.

It is also interesting to note that the tests for H_2S and indole (both substrates contained in the same disk) gave the largest number of variable reactions among triplicate disks inoculated simultaneously; i.e., the reaction of the disk differed from that of the other two disks (Table 3). Of a total of 1,360 triplicate sets of disks (including levulose), 125 (9.2%) of the sets gave variable reactions with MIB inocula, whereas 82 (6.0%) gave variable reactions with LDB inocula. If indole and H_2S results were excluded, 67 (5.6%) of the sets showed variability with MIB inocula, whereas 41 (3.4%) showed variability with LDB inocula. Disk variability was reduced for ten tests and increased for three tests (mannitol, esculin, and mannose) when LDB inocula were used instead of MIB inocula.

Instances were noted in which Minitek results agreed with previously reported conventional test results (3, 6, 8), but the CDC conventional tests did not. On the other hand, in some of the Minitek tests certain strains gave aberrant results in comparison with the reactions reported for conventional tests with that species. When MIB was used, those organisms that were particularly troublesome were four of the four strains of *Clostridium sordellii*, *C. tertium*, *Propionibacterium acnes*, *Bacteroides fragilis* subsp. *vulgatus*, and *Fusobacterium mortiferum* and three of the four strains of *C. septicum* (Table 4). Essentially all of the aberrant results obtained with these organisms were related to the absence of fermentation of various carbohydrates. The major sources of disagreement when LDB was used were the indole and esculin tests. Three of the four strains of *F. nucleatum*, one of the four strains of *B. fragilis* subsp. *fragilis*, one of the four strains of *B. fragilis* subsp. *distasonis*, and one of the four strains of *P. acnes* gave aberrant indole reactions, whereas three of the four strains of *P. granulosum* gave aberrant esculin results. The strains showing aberrant carbohydrate fermentation reactions with LDB were one of four *C. septicum*, two of four *B. fragilis* subsp. *distasonis*, and the single strain of *F. varium*. The biochemical reactions of the organisms in question, when retested by the Minitek procedure, were found to be consistently reproducible, but differed from those with the corresponding CDC conventional tests.

TABLE 3. *Variability of reactions within triplicate[a] sets of Minitek disks*

Substrate or test	No. of triplicate sets	No. of sets showing variability[b] with MIB	No. of sets showing variability[b] with LDB
H_2S	80	33	28
Indole	80	25	13
Mannose	80	4	7
Esculin	80	1	5
Maltose	80	11	5
Salicin	80	4	4
Mannitol	80	2	3
Trehalose	80	5	3
Xylose	80	4	3
Arabinose	80	3	2
Glucose	80	9	2
Rhamnose	80	2	2
Sucrose	80	8	2
Levulose	80	9	1
Glycerol	80	1	1
Nitrate	80	1	1
Lactose	80	3	0
Total	1,360	125	82
Total excluding H_2S, indole	1,200	67	41

[a] Three disks per set, inoculated simultaneously with the same cell suspension.

[b] Number of sets in which the reaction of one of the three disks differed from that of the other two.

The results obtained for the 80 test strains suspended in LDB, at a McFarland no. 5 cell density, and for a 48-h incubation period are shown in Table 5, with the exclusion of tests for H_2S, indole, and nitrate reduction. Superscripts designate the reaction in 12 to 25% of the disks. For example, the triplicate testing of four strains of *B. fragilis* subsp. *thetaiotaomicron* for salicin fermentation resulted in negative reactions for ten disks and positive reactions for two disks. Therefore, since 16.7% (2/12) were positive, the reaction is represented by $(-^{+})$ in the table. Fermentation of salicin by the four strains of *B. fragilis* subsp. *distasonis* was found to be variable because more than one-fourth but less than three-fourths of the disks gave negative reactions. Although the total number of strains tested was small, in only a limited number of instances did results for certain species disagree with reactions previously reported for these species, as judged from tables prepared from composite reactions.

DISCUSSION

The concept of using paper disks impregnated with biochemical substrates as a microtechnique for characterization of bacteria was shown to be feasible by Soto (16) and by Snyder et al (15). Various factors involved in the devel-

TABLE 4. *Reactions obtained with the Minitek system that differed from composite results of conventional tests reported previously*[a]

MIB				LDB			
Species	No. aberrant/no. tested	Substrate or test	Aberrant reaction	Species	No. aberrant/no. tested	Substrate or test	Aberrant reaction
Bacteroides fragilis subsp. *vulgatus*	4/4	Rhamnose	−	*F. nucleatum*	3/4	Indole	−
Clostridium sordellii	4/4	Glucose	−	*P. granulosum*	3/4	Esculin	(+)
		Maltose	−	*B. fragilis* subsp. *distasonis*	1/4	Mannitol	+
C. septicum	3/4	Glucose	−			Indole	+
		Lactose	−	*B. fragilis* subsp. *distasonis*	1/4	Mannitol	+
C. tertium	3/4	Glucose	−	*B. fragilis* subsp. *fragilis*	1/4	Indole	+
		Mannitol	−	*C. septicum*	1/4	Sucrose	+
		Lactose	−			Glycerol	+
		Sucrose	−	*F. mortiferum*	1/4	Nitrate	+
		Maltose	−	*F. varium*	1/1	Xylose	+
		Salicin	−	*P. acnes*	1/4	Indole	−
		Mannose	−			Nitrate	−
Propionibacterium acnes	3/4	Glucose	−				
		Glycerol	−				
		Mannose	−				
Fusobacterium mortiferum	2/4	Glucose	−				
		Lactose	−				
		Mannose	−				
		Esculin	−				
B. fragilis subsp. *distasonis*	1/4	Indole	+				
C. ramosum	1/4	Glucose	−				
		Lactose	−				
		Sucrose	−				
		Salicin	−				
		Mannose	−				
		Trehalose	−				
C. tertium	1/4	Glucose	−				
		Mannitol	−				
		Maltose	−				
		Salicin	−				
		Mannose	−				
F. mortiferum	1/4	Lactose	−				
		Mannose	−				
		Esculin	−				
F. mortiferum	1/4	Lactose	−				
		Esculin	−				
F. nucleatum	1/4	Indole	−				
P. acnes	1/4	Glucose	−				
		Mannitol	−				
Streptococcus intermedius	1/4	Mannose	−				
		Esculin	−				

[a] Dowell and Hawkins (6) and Holdeman and Moore (8). Symbols: +, at least two of three disks showed positive or acid reaction; −, at least two of three disks showed negative reaction; ( ), weak.

opment of rapid microbiochemical tests and the advantages of the use of micromethods in the characterization of bacteria were discussed by Buissiere and Nardon (4) and by Weaver (18). It was emphasized that the use of a large inoculum of bacterial cells from a rapidly growing culture in a small volume of substrate allows rapid biochemical reactions to be obtained.

In our study of representative anaerobic bacteria, the use of a large inoculum (McFarland no. 5 standard) and a new suspension medium helped to improve agreement between the Min-

TABLE 5. *Biochemical reactions[a] obtained with the modified Minitek system*

Species tested (No. of strains used)	Substrate													
	Levulose	Glucose	Mannitol	Lactose	Sucrose	Maltose	Salicin	Glycerol	Xylose	Arabinose	Mannose	Rhamnose	Trehalose	Esculin hydrolysis
Clostridium perfringens (4)	+	+	−	+	+	+	−	+	−	−	+	−	+⁻	+⁻
C. ramosum (4)	+	+	+	+	+	+	+	−	−	−	+	−	+	+
C. septicum (4)	(+⁻)	+	−	+	(v)	+	+⁻	(−⁺)	−	−	v	−	+	+
C. sordellii (4)	+	+	−	−	−	+	−	−	−	−	−⁺	−	−	−
C. sporogenes (4)	v	+	−	−	−	+	−	−	−	−	−	−	+	+
C. tertium (4)	+	+	+	+	+	+	+	−	+	−	+	−	+	+
Bifidobacterium eriksonii (4)	+	+	+	+	+	+	+	−	+	+	+	−	+	+
Propionibacterium acnes (4)	+	(+⁻)	−⁺	−	−	−	−	+	−	−	+	−	−	−
P. granulosum (4)	+	+	−	−	+	+	−	+	−	−	+	−	+⁻	(v)
Bacteroides fragilis subsp. *fragilis* (4)	+	+	−	+	+	+	−	−	+	−	+	−	−	+
B. fragilis subsp. *thetaiotaomicron* (4)	+	+	−	+	+	+	−⁺	−	+	+	+	+⁻	+⁻	+
B. fragilis subsp. *vulgatus* (4)	+	+	−	+	+	+	−	−	+	+	+	(+⁻)	−	−
B. fragilis subsp. *distasonis* (4)	+	+	(v)	+	+	+	v	−	+	v	+	+⁻	+	+
Fusobacterium mortiferum (4)	+	+	−	+	+	v	−⁺	v	−⁺	−	+	−	v	+
F. nucleatum (4)	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Streptococcus intermedius (4)	+	+	+⁻	+	+	+	+	−	−⁺	−	(+⁻)	(v)	+	+
Veillonella parvula (4)	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Eubacterium lentum (4)	−	−	−	−	−	−	−	−	−	−	−	−	−	−
F. necrophorum (3)	v	−⁺	−	−	−	−	−	−	−	−	−	−	−	−
Peptococcus asaccharolyticus (3)	−	−	−	−	−	−	−	−	−	−	−	−	−	−
F. varium (1)	+	+	−	−	−	−	−	−	(+)	−	+	−	−	−
Peptostreptococcus anaerobius (1)	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] All tests were performed in triplicate. Symbols: +, Positive reaction (acid) for 89 to 100% of disks tested; −, negative reaction for 89 to 100% of disks. Superscript indicates the reaction in 12 to 25% of the disks. v, Variable reaction; ( ), different reaction expected according to previous reports (6, 8).

itek tests and conventional tests. These results agree favorably with those of others (4, 12) who found that an inoculum of 10^9 to 10^{10} organisms per ml was optimal for certain microbiochemical tests. A bacterial suspension corresponding to the density of a McFarland no. 5 standard contains approximately 1.5×10^9 organisms (*Escherichia coli*) per ml (2). We feel that it would be impractical, especially in clinical laboratories, to use a cell suspension exceeding the density of a McFarland no. 5 standard for inoculating the Minitek tests. The volume of cell suspension needed to perform 20 Minitek tests is approximately 1.0 ml. Attaining a McFarland no. 5 cell density in this small volume is quite feasible in most instances, assuming that the cell suspension is prepared from a 48-h anaerobic BA culture derived from a single colony isolate. In our study, sufficient growth of all anaerobes except *Eubacterium lentum* was obtained by this method. Cultures of *E. lentum* may require 72 h of incubation or longer in order to supply enough cells on a single BA plate. All procedures except incubation can be performed on the bench top in ambient atmospheric conditions.

The modified recommendations of the manufacturer for interpretation of color reactions obtained with the Minitek tests were very helpful and undoubtedly improved the reproducibility of results recorded by different readers. These recommendations eliminated disagreement

over the interpretation of colors varying between yellow and orange and increased the speed in reading the results.

The decision to exclude the Minitek urea disk from the study was based on the observation that *C. sordellii,* one of the few urease producers among the anaerobes, tended to produce strong alkaline changes in other disks adjacent to the urea well even though the urea disk was overlayed with mineral oil.

Although the results obtained with the carbohydrate-free thioglycolate broth inocula were comparable to those obtained with the LDB inocula, there was less reduction of the indicator by microorganisms in the LDB. For this reason the LDB was selected for subsequent testing with a larger number of bacterial strains. The 22 species of anaerobes used in the expanded study were selected because these, with the exception of *F. varium,* are commonly isolated from clinical specimens. Isolates from specimens likely to be contaminated with normal flora were deliberately excluded from the study.

Traditionally, microbiochemical tests for differentiation of bacteria have been evaluated by determining the degree to which their results agree with results of corresponding conventional tests (4, 7, 9, 11, 13, 14, 17). We were able to modify the Minitek system so that most of the Minitek substrates tested gave reproducible results that agreed favorably with results of analogous conventional tests. We have designated the procedure of using Minitek disks and an inoculum prepared in LDB equal to a McFarland no. 5 standard as the "modified Minitek system." With the exception of the tests for indole, H_2S, and reduction of nitrate, the overall agreement of the Minitek tests (carbohydrates and esculin) with conventional tests was better than 95%. We found that 13 of the 80 strains (16.3%) tested in stage 2 could have been misidentified if modified Minitek results were compared with reactions previously reported (Table 4). The percentage of strains giving aberrant results was reduced to 8.8% if the indole and nitrate results were obtained by conventional procedures. The strains producing an aberrant result for carbohydrate fermentation or esculin hydrolysis were three of four *P. granulosum,* two of four *B. fragilis* subsp. *distasonis,* one of four *C. septicum,* and the single strain of *F. varium.*

It is doubtful that micromethod reactions will ever agree completely with those of conventional tests. The reactions for a species listed in taxonomic tables (3, 6, 8) are the composite results obtained when specific conventional procedures are used in the examination of multiple strains of that species. The symbols (+) and (−) do not necessarily signify 100% agreement for that character (the customary range being 90 to 100%). In addition, as others have pointed out, because the volumes of substrates are reduced in microtechniques, the buffering capacity of the substrate, the type of indicator used, the oxidation-reduction potential, the proportion of inoculum to substrate, and the age of the inoculum become more critical in their influence on the reactions (4, 14). It is quite probable that reactions with certain micromethod tests will be different from those with the corresponding conventional tests. However, the reactions obtained with the micromethod tests will be valid if they are consistent and reproducible in tests with a large number of different strains of the same species. For this reason, we feel that data from the examination of various microorganisms with each particular micromethod should be compiled and used to prepare separate differential tables and keys for each individual system.

Although we inoculated three disks of each substrate simultaneously in order to assess substrate variation from disk to disk in the same lot of disks, additional factors such as contamination of the wells with other bacteria and strain or species variability in the production of specific enzymes may also have influenced the results. The conventional substrates were not tested simultaneously in triplicate, but the test strains had all been characterized with conventional tests at least once before they were used in our study, and all were retested from the same pure culture used to inoculate the Minitek tests. Any discrepancies between the two sets of conventional test results were rechecked a third time.

Because of the extreme variability of the results obtained with the Minitek H_2S-indole disks, independent of the type of suspension medium or microorganism tested, and because the conventional tests for indole and H_2S showed essentially no variability, we feel that the H_2S-indole disks are unacceptable for use with anaerobes. The remaining instances of varying reactions within triplicate sets of Minitek disks were frequently associated with a particular species or subspecies reported to exhibit variable reactions with the analogous conventional test (6, 8). If these variable reactions are excluded, the number of sets of disks with variable reactions is reduced from 67 to 34 with MIB and from 41 to 20 for LDB suspension medium out of a total of 1,200 sets tested (Table 3).

Levulose (fructose) and glucose are metabolized in a similar but not identical manner (5). Because of the initial problems with dextrose-nitrate disks, levulose fermentation was also tested by the Minitek procedure. There was 79.6% agreement between Minitek levulose fermentation and conventional glucose fermentation with MIB inocula and 90.4% agreement with the LDB inocula.

The rapidity of reactions by the modified Minitek procedure was studied with a limited number of cultures. A representative strain of each of 20 species (common clinical isolates) was selected at random, and the cultures were examined after 24 h and after 48 h of incubation. It was found that the majority of the reactions were complete after 24 h, but exceptions were noted, particularly with certain organisms tending to reduce the phenol red indicator, such as *C. septicum*. If additional dilute phenol red indicator is added to the disks to allow the reactions to be read after reduction of the indicator by the culture, the test should not be reincubated. Also, since we do not know the effect of reincubation after the disks are exposed to air, we recommend a single and final reading of the Minitek tests at 48 h when GasPak jars are used. However, we have found in another study that the Minitek tests can be incubated in an anaerobic glove box if a high relative humidity is maintained to prevent drying of the disks. The use of a glove box allows the reactions to be observed as frequently as desired without exposure to air.

Preliminary results with a variation of the Minitek indole test have been encouraging. Twenty-six strains of anaerobes representing 20 species were used to compare the new technique with the original disk test for indole. The new test is performed by adding approximately 0.10 ml of the cell suspension to a Minitek well without a disk. After incubation, Kovac reagent is added. Two strains of *F. necrophorum*, one *F. nucleatum*, and one *P. acnes* were indole negative by the original disk test but positive by both the conventional procedure and the new "disk-free" technique. One strain of *B. fragilis* subsp. *thetaiotaomicron* gave a false negative reaction by both the disk and disk-free techniques, whereas one strain of *C. sporogenes* showed a false positive indole reaction by the disk-free technique alone.

A limited number of strains have been used to test additional variations of the modified Minitek tests for esculin hydrolysis and nitrate reduction as performed in stage 2. Improved correlation with conventional esculin hydrolysis results has been obtained by confirming any questionable Minitek esculin reactions by examination for fluorescence of the esculin disk with a Wood's lamp. The three strains of *P. granulosum* giving aberrant weakly positive reactions for esculin hydrolysis by the modified Minitek procedure (Table 4) showed negative reactions for esculin hydrolysis by the fluorescence test (no decrease in intensity of fluorescence when compared with a negative control). A Wood's lamp can be used in the glove box if it is desired to read esculin hydrolysis results without exposing the culture to air. A few of the false negative Minitek results for nitrate reduction could have been due to the formation of reduced products other than nitrite. We have found that the addition of a pinch of zinc dust to those nitrate tests remaining yellow after nitrate A and B reagents are added has eliminated some of the aberrant results. The color of the nitrate disk remains bright yellow even after the zinc dust is added to Minitek nitrate tests of *C. perfringens* cultures, which suggests that the organism reduces nitrate to substances other than nitrite. However, occasional cultures of other species negative for nitrate reduction when tested by the conventional procedure exhibited false positive nitrate reduction when zinc dust was added to the Minitek disk to test for residual nitrate.

A small number of strains have been tested for decomposition of hydrogen peroxide by adding 3% H_2O_2 to a Minitek well containing cell suspension but no disk. Overall the results obtained were consistent with tests performed by the conventional technique. We have noted that *B. fragilis* strains consistently exhibit active decomposition of H_2O_2 when tested in the Minitek plates using LDB. It has also been found that, at least for *B. fragilis* strains, the H_2O_2 decomposition is weaker if the H_2O_2 is added to a well containing a carbohydrate fermented by the organism.

An alternative procedure for obtaining a suitable inoculum for the Minitek system has been tested with 53 strains of anaerobes representing 14 species. This procedure consists of inoculating a tube of the LDB without added carbohydrate from a single colony on a BA culture. The broth culture is incubated for 18 to 24 h or until evidence of growth is detectable by any increase in turbidity, and then aliquots of the broth are dispensed into the Minitek wells containing appropriate disks. Preliminary results suggest that results of the fermentation tests performed with this procedure are as reliable as those obtained with the procedure using a cell suspension prepared from a BA culture. An additional advantage is the decreased probability of contaminating a pure culture.

One of the major advantages of the Minitek

system is its flexibility and versatility in allowing a variety of substrates to be selected for testing. We have found that the Minitek tests are simple to perform and easily interpreted. Supplies for the system require less storage space than conventional tubed media, the total volume of media and glassware necessary for biochemical characterization of anaerobes is reduced, and disposal of the Minitek plates after autoclaving presents no problems. For those laboratories preparing their own media, use of the Minitek system effectively reduces preparation time. Also, once a pure culture derived from a single colony isolate is obtained, the Minitek system allows a savings of 3 to 5 days over the conventional system for determining the fermentation of carbohydrates, assuming that the final reading of the Minitek system is made at 2 days versus 5 to 7 days for the conventional system.

One disadvantage of the Minitek system that we noted in our studies was the proclivity for mechanical breakdown of the pipetter and variations in volume dispensed by different pipetters. However, the inoculum can be successfully dispensed with a sterile Pasteur pipette or a serological pipette if necessary. Another disadvantage of the Minitek system is the number of disk-dispensing devices and Minitek plates required for a panel of biochemical tests. The disk dispenser holds only 10 substrate cartridges, and the Minitek plate contains only 12 wells. If more than 12 tests are desired, at least two of the Minitek plates are required.

We have concluded that the modified Minitek system is a useful and acceptable substitute for the more expensive, time-consuming conventional tests for determining carbohydrate fermentation and esculin hydrolysis by anaerobes. The results of useful differential tests can be obtained within 48 h after adequate growth of a pure culture is obtained. When used in conjunction with other conventional tests, such as those for lecithinase, lipase, growth in the presence of 20% bile, susceptibility to penicillin, and gas-liquid chromatography profiles of metabolic products (the results of which can usually be obtained after 48 h of incubation), this micromethod system can effectively aid in reducing the time and expense required for identification of anaerobes to the species or subspecies level.

ADDENDUM

We recently evaluated an experimental Minitek anaerobic broth that was supplied to us by BBL after the study was concluded. According to the manufacturer, the formulation of the Minitek anaerobic broth corresponded to that of LDB as reported by us at the 75th Annual Meeting of the American Society for Microbiology (Abstr. C34, p. 32, 1975). Cell suspensions were made from pure BA cultures of 43 strains of anaerobes representing 24 species or subspecies in both the experimental Minitek anaerobic broth and LDB prepared in our laboratory. Parallel sets of eight different carbohydrate disks and an esculin disk were inoculated with the two suspensions of each organism. Only 5 of the 387 tests performed (1.3%) resulted in differing reactions with the two broths.

A prototype disk for testing nitrate reduction was evaluated with these 43 strains. The experimental Minitek anaerobic broth was used as the suspending medium. One strain of *Peptostreptococcus anaerobius* showed a positive reaction with the original dextrose-nitrate disk and a negative reaction with the prototype nitrate disk. Four instances were noted in which failure to demonstrate residual nitrate by addition of zinc dust resulted in positive reactions with the original disk; in contrast, rapid appearance of red coloration confirmed residual nitrate in the prototype disk after zinc dust was added to the tests for these organisms. The only reactions that could have resulted in misidentification of organisms were false positive reactions by two *B. fragilis* subspecies, both of which occurred only with the original disk; the other discrepancies between the two disks mentioned above corresponded to reactions previously reported to be variable for that particular species.

The production of indole by these 43 strains was tested by three methods with aliquots of the same cell suspension prepared in the experimental Minitek anaerobic broth and dispensed in 0.1-ml amounts to separate wells of the Minitek plate. After incubation, 2 drops of Kovac reagent was added to one of the wells. To another, 2 drops of xylene followed by 2 drops of Kovac reagent was added. A portion of the broth in the third well was removed with a Pasteur pipette, and a drop was applied to a disk of filter paper saturated with *p*-dimethyl- aminocinnamaldehyde, as described by Sutter and Carter (V. L. Sutter, and W. T. Carter, Am. J. Clin. Pathol. 58:335-338, 1972). No false positive reactions occurred with any of the three methods. Two false negative reactions were noted, both occurring in the procedure using Kovac reagent without xylene extraction. In every instance, the red color of the positive indole test with Kovac reagent was enhanced by the xylene extraction.

ACKNOWLEDGMENTS

We would like to acknowledge the assistance of Marie Lee and Jacquelyn Anderson in the preparation of materials and tabulation of reactions.

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