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Bromothymol Blue and Carbohydrate-Sensitive Plating Media

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A new plating medium using bromothymol blue (BTB) indicator is described and compared with eosin-methylene blue (EMB), MacConkey, and Endo media. These media were tested with L-arabinose by plating fermenting and nonfermenting mutant strains of *Escherichia coli*. The minimum concentrations of L-arabinose that permitted differentiation of these strains were determined. Different concentrations were required for differentiating confluent patches of cells, isolated colonies, and closely spaced or adjacent colonies. L-Arabinose, L-rhamnose, D-lactose, and D-galactose were tested with modified enteric media and with BTB medium, again to determine minimum usable concentrations. BTB media and reformulated conventional media allowed detection of acidification, aerobically, at one-fifth to one-hundredth the (1%, wt/vol) concentration of carbohydrate used in standard indicator plates.

Commonly used plate differential media were originally formulated to provide rapid growth of nonfastidious bacteria, to select against bacteria that were not of interest, and to detect acid production, generally from D-lactose with or without sucrose. To provide rapid growth in the presence of inhibitory compounds or dyes, it was necessary to use high concentrations of one or more peptones. MacConkey agar (10), Endo agar (3, 5, 6), and eosin-methylene blue (EMB) agar (7) use 20, 10, and 10 g of peptone per liter, respectively. Carbohydrates have generally been used at a concentration of 1% (wt/vol) to detect acidification against the alkalinization caused by peptone catabolism.

The medium of Hugh and Leifson, OF medium, was devised to detect weak, oxidative saccharolytic activity of nonfermentative bacteria (8). This medium incorporates 0.2% peptone, 1% carbohydrate, and bromothymol blue (BTB) indicator, and is designed for maximum sensitivity in assaying acid production in tubes. OF medium, like Endo and EMB media, uses a phosphate buffer.

It would be beneficial to have a plating medium that is more sensitive to acidification and yet more economical than conventional media. Such a medium might be useful for one or more of the following purposes: (i) selection of fast-growing bacteria aerobically or anaerobically; (ii) alleviation of carbohydrate inhibition caused by partial metabolism of certain carbohydrates; (iii) more sensitive determination of acid production by weakly saccharolytic species; and (iv) differentiation with a specific alkaline-reacting substrate alone or in conjunction with a sugar, without masking of the pH reaction by peptone. The formulas given in this paper will allow reasonable aerobic growth of *Escherichia coli* with increased pH sensitivity at low carbohydrate and peptone concentrations. Conclusions concerning the proportion of peptone to carbohydrate and the concentrations of indicators and bile salts should be applicable to other enteric media and nonfastidious bacteria.

MATERIALS AND METHODS

Abbreviations. Percentage of a solid ingredient refers to grams dissolved in 100 ml, final volume, of solution (aqueous unless otherwise noted). Percentage of a liquid refers to volume (milliliters in 100 ml) percent. Modified formulations of MacConkey, EMB, and Endo media are designated Mac-S, EMB-S and Endo-S. BTB refers to the less carbohydrate-sensitive (0.2% tryptone) BTB formula; BTB-S refers to the more sensitive formula; BTB media refers to both media.

***E. coli* strains.** All bacterial strains were *E. coli* K-12. K-12Δ766 (F⁻K-12 514 *araΔ766 Δlac str*^{r}) was constructed by P1 transduction of strain F⁻K-12 514 *leu str*^{r} as described by Wilcox et al. (14) and served as a nonfermenter control for lactose and arabinose. An L-rhamnose-negative derivative of K-12Δ766, *rha-313*, was obtained by nitrosoguanidine mutagenesis. AB1203, F⁻K-12 *ilv arg gal lac xyl T4*^{r} *T6*^{r}, has been described by Pittard and Ramakrishnan (12). P4X, Hfr K-12 *metB argE thi*, served as wild type (9).

Media. All peptones, beef extract, yeast extract, bile salts, agar, EMB agar base, and MacConkey agar base were Difco products. NaCl and Na_2SO_3 were from J. T. Baker Chemical Co., and K_2HPO_4 was from Mallinckrodt Chemical Works. BTB was bought from National Chemical Labs; basic fuchsin, methylene blue, and eosin Y were bought from Allied Chemical Corp.; and neutral red was bought from Matheson,

Coleman & Bell. L-Rhamnose was from Sigma Chemical Co.; L-arabinose and D-galactose were from Calbiochem; and D-lactose was from Nutritional Biochemicals Corp.

Medium preparation. For each batch of media, solid constituents were suspended in 80 to 90% of the final volume of water, and this basal medium was autoclaved for 15 min at 15 lb/in^2. Sterile salt, dye, and carbohydrate solutions were then added (BTB solution, 0.3% in 100 mM NaOH, is not sterilized but added to the basal medium before autoclaving). The volume was adjusted in a sterile, graduated cylinder with sterile distilled water. From 100 ml of medium, three or four plates were poured.

Sugars, EMB solution, and basic fuchsin were sterilized with a membrane filter apparatus (Millipore Corp.). Sugar solutions were prepared at a concentration of 0.5, 1, or 10%. Per mole of sugar, the degree of hydration was as follows: L-arabinose, 0; L-rhamnose, 1; D-lactose, 0; and D-galactose, 0. For equal weight percent solutions, molarities were in the proportions, respectively, of 1:0.82:0.44:0.83. EMB solution was 2% in eosin Y and 0.325% in methylene blue. Basic fuchsin was a 5% solution in 95% ethanol. Neutral red (0.1%) and a 10% solution of anhydrous Na_2SO_3 were autoclaved for 15 min at 15 lb/in^2.

Conventional MacConkey and EMB agars were prepared from Difco basal media (refer to Table 1). Conventional Endo, Endo-S, Mac-S, and EMB-S media were prepared from components. Endo plates were not used until the light-induced development of a sufficiently red color had occurred. This occurs uniformly in partially covered boxes in which plates receive indirect fluorescent illumination. The coloration occurs less uniformly in direct fluorescent illumination (3 to 7 days) and in direct sunlight (a few hours). The color sought lies between Ridgway's amarynth purple and pomegranate purple (plate XII in 13). The plates, although not opaque, were a darker red than is shown in a book by Evans (plate VIII in 4) and in the article by Nambu (region "G"; 11).

Two formulations of BTB medium are given. BTB basal is prepared by dissolving tryptone, NaCl, and BTB solution in a 90% volume of water at room temperature, adding 10 N HCl dropwise until a yellowish-green color results, and then adding bile salts and agar. The color is best viewed by drawing the medium into a 10-ml pipette. Bile salts and agar tend to raise the pH; heat and acidic carbohydrates lower the pH. Final pH is 6 to 6.5.

Plating and scoring procedure. To test for differentiation, a plate was divided in half and streaked as follows. Inocula of one fermenting and one nonfermenting strain were taken from nutrient broth deeps. The inocula were streaked in 2-cm^2 patches, side by side, at one end of the plate. Each patch was streaked out separately so that cells of each inoculum were diluted out on separate halves of the plate. The dilute end of the plate was then inoculated with a mixture of the two strains and streaked back toward the separately inoculated end of the plate. Three types of responses were scored: (i) the pH reactions of the confluent regions (cells of the separately inoculated patches); (ii) the pH reactions of isolated colonies; and (iii) the pH reactions of adjacent or closely spaced colonies. Plates were observed in random order, and the ability of a medium to differentiate was judged, respectively, by: (i) the color of cells in a patch or its periphery; (ii) the colors of colonies that were separated by more than 2 mm; and (iii) the occurrence of touching colonies of opposite reaction.

BTB media and MacConkey plates were incubated for 16 to 20 h; EMB and Endo plates were incubated for 24 to 30 h. The respective colony colors were: (i) acid reaction (yellow, red, purple [with or without greenish sheen], and red [with or without bronze sheen]); and (ii) alkaline reaction (blue-gray, yellow-white, gray, and pink-white).

TABLE 1. *Formulations of media*[a]

Ingredient	Amt in given medium							
	BTB	BTB-S	Mac-Conkey	Mac-S	EMB	EMB-S	Endo	Endo-S
Tryptone	2	0.5	0	0.5	0	0.5	0	1
NaCl	5	5	0	5	0	0	0	0
BTB solution[b]	30	60	0	0	0	0	0	0
Neutral red solution	0	0	0	30	0	0	0	0
EMB solution	0	0	0	0	0	20	0	0
Basic fuchsin solution	0	0	0	0	0	0	10	10
Na_2SO_3 solution	0	0	0	0	0	0	25	5
Bacto-peptone	0	0	0	0	0	0	10	0
K_2HPO_4	0	0	0	0	0	0	3.5	0
MacConkey agar base	0	0	40	0	0	0	0	0
EMB agar base	0	0	0	0	27.5	0	0	0
Bile salts no. 3	3	3	0	3	0	0	0	3
Agar	15	15	0	15	0	15	15	15
Water[c]								
Carbohydrate solution[c]								

[a] Solutions are added after autoclaving the basal medium and cooling to 55°C. Numbers represent grams of ingredient or, for solutions, milliliters.

[b] Neutralized in the basal medium, by addition of concentrated HCl, before addition of bile salts and agar.

[c] See Materials and Methods.

RESULTS

Development of BTB agar. Difco purple agar was the starting point for the development of a new medium capable of detecting weak acid production (2). This formula was tested with bromocresol purple (0.002%), phenol red (0.0025%), Andrade indicator (1%), and BTB (0.0033%), using 0.3% L-rhamnose. Only BTB showed clearly opposing colony colors as well as opposing reactions on the plate surface. After the concentration of peptone was reduced to 0.5%, nine different Difco peptones were tested with 0.2% L-rhamnose. Casein peptones, which contain less occluded carbohydrate than beef peptones, were expected to provide better differentiation. Tryptose and tryptone proved satisfactory, and 0.5% tryptose was used for tests of certain components (Table 2, footnote *e*). Bile salts, which precipitate in acid to a grayish-yellow color, enhanced the yellow acid color of BTB. The OF formula, which uses BTB and tryptone, suggested a lower concentration of peptone. Tryptone at the OF concentration of 0.2%, without beef extract, differentiated closely spaced colonies.

The results shown in Table 2 represent initial tests with three formulations of BTB medium (refer to footnotes, Table 2). Different concentrations of individual components were tested. The concentration giving the best adjacent-colony differentiation with limiting carbohydrate was determined and incorporated into the BTB medium formulas. Limiting concentrations shown are those nearest to the optimum that were tested and found to give poorer differentiation.

Tests with BTB and with BTB-S media suggested a tryptone concentration less than 0.1%. The lower limit was determined by the ability to show colonies after a short enough period of time that (i) acid was not thoroughly oxidized and (ii) the difference in size between acid producers and nonproducers was not great. A concentration of 0.05% in BTB-S agar gave colony sizes between 0.5 and 2 mm after 18 h.

BTB was found to work well at 0.09 or 0.18 g/liter. Lower concentrations did not show acid as well, and higher concentrations did not enhance differentiation. Bile salts no. 3 used at the concentration of MacConkey plates (0.15%) did not show as good a difference in colony color as with 0.3%. With concentrations of 0.45% or greater, differentiation was rapidly lost due to excess precipitation. BTB medium gave poor differentiation with 5% agar and none with 0.5% yeast extract.

Responses of conventional media and BTB media with L-arabinose. The two formulas for BTB media, BTB-S and BTB, were

TABLE 2. *Limiting concentrations of components of BTB medium*

Component	Concn	Tested
Tryptone[a]	≤0.25 g/liter[b]	0.1, 0.25, 0.5 g/liter
	≥1 g/liter[c]	0.5, 1, 2 g/liter
BTB[d]	≤0.033 g/liter[b,c,e]	0.033, 0.09, 0.18, 0.3, 0.5 g/liter[b,c]; 0.033, 0.1, 0.5 g/liter[e]
	≥0.5 g/liter[b,c,e]	0.033, 0.09, 0.18, 0.3, 0.5 g/liter[b,c]; 0.033, 0.1, 0.5 g/liter[e]
Bile salts no. 3[f]	≤0.15%[b,c,e]	0.15, 0.3, 0.45, 0.6%[b,c]; 0.15, 0.3, 1.5%[e]
	≥0.45%[b,c]	0.15, 0.3, 0.45, 0.6%;

[a] Lowest suitable concentration of tryptone was that which allowed reasonable aerobic growth by 24 h. The 0.01 and 0.025% tryptone gave isolated colonies of 0.5- to 1-mm diameter, with arabinose-negative colonies generally smaller than arabinose-positive colonies; at 0.05%, colonies were 0.5 to 2 mm. The 0.01 and 0.025% tryptone gave slightly better differentiation than 0.05% with 0.025% L-arabinose and identified isolated arabinose-positive colonies at 0.01% L-arabinose. No growth could be achieved by anaerobic incubation for 1 week of K-12Δ766 on 0.01% tryptone BTB-S plates.

[b] Tests were done with BTB-S formulation and 0.025% L-arabinose.

[c] Tests were done with BTB and 0.05% L-arabinose.

[d] Lowest concentration of BTB tested gave poorer differentiation than the next-higher concentration. Increasing concentrations showed no increase or decrease in differentiation between L-arabinose-positive and -negative colonies, although arabinose-positive colonies stood out more against the background color of the plate with higher concentrations of BTB.

[e] Tests were done with BTB, substituting tryptose as peptone at 0.5% and using 0.1% beef extract, 0.2% L-rhamnose, and 0.15% bile salts no. 3.

[f] Optimum concentration of bile salts no. 3 that enhanced the color reaction lay between 0.15 and 0.45%. In differentiating colonies at threshold sugar concentrations, the opacity of colonies when viewed by transmitted light aids in differentiation.

chosen for further tests based on (i) ability to show acid at a very low carbohydrate concentration (hence, 0.05% tryptone) with positives able to stand out readily (hence, 0.18 g of BTB per liter) and (ii) ability to show acid at a relatively low carbohydrate concentration but with faster growth (hence, 0.2% tryptone, with 0.09 g of BTB per liter for economy). These media differentiated acid production at lower carbohydrate concentrations than did conventional media (refer to Table 3). Incubation time was important in the ability of MacConkey agar and BTB media to differentiate at threshold concentrations. MacConkey agar faded to the alkaline, yellow color by 24 h, and BTB plates, if heavily inoculated, became blue by the same time.

Endo plates turn deep red when left out in the light (5). When they were used fresh, differentiation was poor; if they turned too deep a red, growth was inhibited. The shade used was arrived at after comparing differentiation with the conventional formula, and in subsequent tests this color was retained. EMB plates, like Endo plates, did not fade readily, although EMB did not appear to be as sensitive as properly prepared Endo agar.

TABLE 3. *Minimum concentrations of L-arabinose permitting a given type of differentiation: BTB media and conventional MacConkey, Endo, and EMB media*[a]

Medium	Confluent regions (%)	Isolated colonies (%)	Adjacent colonies (%)
BTB	0.05	0.05	0.05
BTB-S	0.025	0.025	0.025
MacConkey	0.2	0.1	0.2
Endo	0.1	0.1	0.2
EMB	0.4	0.2	0.2

[a] BTB and conventional media with varying concentrations of L-arabinose were streaked and scored by criteria described in the text. Concentrations tested were: 0.01, 0.025, 0.05, and 0.1% (BTB media); and 0.1, 0.2, 0.4, and 1% (conventional media). Concentrations reported are the lowest concentrations for which a difference in reaction was observed by the above criteria.

Responses of modified enteric media and BTB media with various sugars. Experiments with components of BTB media suggested reformulation of conventional media. Conventional media were altered with respect to buffering capacity, presence of bile salts, and type and concentration of peptone (refer to Table 1). The results with 0.05% L-arabinose are summarized below. Tryptone was chosen as the peptone for these media. A tryptone concentration of 0.05% provided better differentiation than 0.1% for Mac-S and EMB-S. Endo-S with 0.1% tryptone was more satisfactory, however, probably because of slowness of growth on Endo agar. Original concentrations of indicators were retained except in Endo formula, where the concentration of decolorizing agent, Na_2SO_3, was reduced. Bile salts no. 3 at 0.3% improved differentiation by Mac-S and Endo-S agar, but prevented differentiation by EMB-S agar with 0.05% L-arabinose. No buffering or pH adjustment of conventional media was used.

The ability of these media to detect acid production from various sugars is shown in Table 4. Endo-S detected acid at the lowest carbohydrate concentrations, especially in differentiation of confluent regions. BTB-S was more sensitive than Endo-S and EMB-S with respect to L-arabinose differentiation, but with respect to other sugars was less sensitive although more quick to develop. Mac-S was the least sensitive of the media and faded very quickly at threshold carbohydrate concentrations.

DISCUSSION

For the purpose of detecting acid production by nonfastidious bacteria, conventional media are wasteful and insensitive in their formulations. *E. coli* grown aerobically on 0.05% tryptone give adequate colony sizes (0.5 to 2 mm) in 16 to 24 h for Mac-S and BTB-S media and in 24 to 36 h for EMB-S medium to permit colony identification. Endo-S requires 0.1% tryptone at threshold carbohydrate concentrations. Bacto-bile salts (Difco) may be substituted in BTB medium for bile salts no. 3, further

TABLE 4. *Minimum concentrations of various sugars: BTB media and modified MacConkey, Endo, and EMB media*[a]

Medium	Confluent regions (%)					Isolated colonies (%)					Adjacent colonies (%)				
	BTB	BTB-S	Mac-S	Endo-S	EMB-S	BTB	BTB-S	Mac-S	Endo-S	EMB-S	BTB	BTB-S	Mac-S	Endo-S	EMB-S
L-Arabinose	0.05	0.025	0.1	0.01	0.025	0.05	0.025	0.025	0.01	0.025	0.05	0.025	0.05	0.05	0.05
D-Lactose	0.1	0.05	0.1	0.01	0.025	0.1	0.05	0.05	0.025	0.025	0.1	0.05	0.1	0.05	0.05
L-Rhamnose	0.1	0.05	0.1	0.01	0.05	0.1	0.05	0.05	0.025	0.05	0.1	0.05	0.1	0.05	0.2
D-Galactose	0.1	0.05	0.1	0.01	0.025	0.1	0.05	0.025	0.025	0.01	0.1	0.05	0.1	0.05	0.05

[a] Media were streaked and observed by criteria described in the text. Sugar concentrations tested were 0.01, 0.025, 0.05, 0.1, and 0.2%.

reducing cost. A compromise BTB medium in use in our laboratory consists of the BTB formula with 0.1% tryptone, 0.15% Bacto-bile salts, and 0.1% carbohydrate. The cost of this medium per liter, using Difco components and 0.1% D-lactose, and the cost per liter of complete MacConkey, EMB, and Endo media are, respectively (1975–76 catalog): $0.93, $1.42, $0.97, and $1.34.

The ability of these media to support the growth of more fastidious or slow-growing bacteria has not been studied. The lack of buffering, which favors detection of small changes in pH, may restrict growth. Where the background is expected to be strongly alkaline, detection of acid may require (i) increased carbohydrate, (ii) buffering, (iii) addition of inhibitors, or (iv) anaerobic incubation. Substitution of 0.09 g of BTB per liter for neutral red in otherwise complete MacConkey agar (1% D-lactose) allows differentiation of *E. coli* wild type from mutants. From the tests with bile salts, it is suggested that enteric media designed to screen for bile-resistant organisms should be retested for greater pH sensitivity vis-a-vis decreased selectivity.

The phenomenon of carbohydrate inhibition due to accumulation of phosphorylated intermediates is seen with several sugars, e.g., galactose, arabinose, rhamnose, and glycerol (1). Use of a medium with lower carbohydrate concentration may allow similar carbohydrate-sensitive phenotypes to be expressed, facilitating screening for these mutations. The use of overlays of mutagenized cells in top agar without carbohydrate might allow better growth of such mutants. Further, permease mutations that are masked by high carbohydrate concentrations may be selectable on such media. It is doubtful that the use of multiple indicators would allow more sensitive differentiation, since the pH change of the more sensitive indicator would be masked by the other indicator. Formulations designed to detect acid anaerobically might be more sensitive due to absence of acid oxidation.

Before attempting to use threshold concentrations of sugars, the media should be checked with positive and negative control cultures on the same plate. This is especially important in using media that fade, or even with Endo agar, which varies in its differentiation capability according to its color. It might be desirable in some instances to screen for nonfastidious bacteria on plates containing two oppositely reacting substrates such as glucose and urea. When a low concentration of peptone is used, the pH reaction of a colony would be primarily determined by its use of one or both substrates.

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LITERATURE CITED

1. **Cozzarelli, N. R., J. P. Koch, S. Hayashi, and E. C. C. Lin.** 1965. Growth stasis by accumulated L-α-glycerophosphate in *Escherichia coli*. J. Bacteriol. **90**:1325–1329.
2. **Difco Laboratories.** 1953. Bacto purple agar base, p. 190. *In* Difco manual of dehydrated culture media and reagents, 9th ed. Difco Laboratories, Inc., Detroit.
3. **Endo, S.** 1904. Ueber ein Verfahren zum Nachweis der Typhusbacillen. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **35**:109–110.
4. **Evans, R. M.** 1948. An introduction to color. John Wiley & Sons, Inc., New York.
5. **Harris, N. M.** 1925. The preparation of Endo's medium. Mil. Surg. **57**:280–285.
6. **Harris, N. M.** 1925. The preparation of Endo's medium—a correction. Mil. Surg. **57**:391.
7. **Holt-Harris, J. E., and O. Teague.** 1916. A new culture medium for the isolation of *Bacillus typhosus* from stools. J. Infect. Dis. **18**:596–600.
8. **Hugh, R., and E. Leifson.** 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative bacteria. J. Bacteriol. **66**:24–26.
9. **Jacob, F., and E. L. Wollman.** 1957. Analyse des groupes de liaison génétique de différentes souche donatrices d'*Escherichia coli* K12. C. R. Acad. Sci. **245**:1840–1843.
10. **MacConkey, A.** 1905. Lactose-fermenting bacteria in faeces. J. Hyg. **5**:333–379.
11. **Nambu, Y.** 1976. The confinement of quarks, p. 54. Sci. Am. **235**:48–60.
12. **Pittard, J., and T. Ramakrishnan.** 1964. Gene transfer by F' strains of *Escherichia coli*. J. Bacteriol. **88**:367–373.
13. **Ridgway, R.** 1912. Color standards and color nomenclature. R. Ridgway, Washington, D.C.
14. **Wilcox, G., P. Meuris, R. Bass, and E. Englesberg.** 1974. Regulation of the L-arabinose operon *BAD in vitro*. J. Biol. Chem. **240**:2946–2952.