Factors Affecting Selectivity of Brilliant Green-Phenol Red Agar for Salmonellae

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Commercial brilliant green (BG)-sulfa agar was found to be nonselective toward a test series of Enterobacteriaceae. Various formulations of BG were prepared by using Trypticase soy agar (BBL) as a base. Results were more reproducible when BG dye was added after sterilization than before. Sulfonamides improved selectivity as compared with brilliant green alone. Sulfanilamide (SN) was slightly more selective for salmonellae than other sulfonamides tested. Bile salts and sodium dodecyl sulfate markedly reduced the toxicity of BG to all the test bacteria. Enterobacter strains were most difficult to inhibit. A combination of 5 mg of BG and 1 g of SN/liter prevented growth of Proteus mirabilis and Escherichia coli and retarded growth of Enterobacter strains. The BG-SN agars were superior in selectivity to a series of commercial agars tested, and numbers of salmonellae recovered on BG-SN agar and Trypticase soy agar (BBL) were the same. Brilliant green agars with various degrees of selectivity are described.

The first satisfactory selective and differential solid medium for salmonellae using brilliant green (BG) was the brilliant green-phenol red agar (BGA) described by Kristensen et al. (9) in 1925. In this formulation BG was incorporated as the selective agent, phenol red was incorporated as an acid base indicator, and sucrose and lactose were incorporated as fermentable carbohydrates. Most bacteria which are not inhibited by the BG ferment either sucrose or lactose and form green colonies; salmonellae produce an alkaline reaction on the medium and form pink colonies. Galton et al. (3) and Osborne and Stokes (13) added sulfadiazine and sulfapyridine, respectively, to the agar to improve the selectivity.

BGA or brilliant green-sulfa agars (BGS) have been found highly satisfactory as plating media for salmonellae. Banwart and Ayres (1) found better recoveries of salmonellae on BGA than on other selective media. Taylor et al. (17) describe BGA as the plating medium of choice for salmonellae. Of seven recommended methods (12) for detection of salmonellae, all use BGA or BGS as one, if not the sole, plating media.

Despite this excellent record, some problems have been noted in controlling the selectivity of BGA and BGS. Hobbs (6) noted that BGA was only slightly selective. Read and Reyes (15) found that some lots of commercial BGA (to which they added sulfadiazine) would not support satisfactory growth of salmonellae.

Lang (10) found that BGA showed satisfactory selectivity only when prepared from the ingredients with the dye added after the medium was sterilized and cooled to 50 C. Some authors recommend autoclaving the agar a specified length of time to attain optimum selectivity. Galton et al. (4) recommend autoclaving 15 min at 121 C, and Poelma (14) recommends 12 min. Galton et al. state that with a shorter time the medium was toxic to salmonellae, whereas with a longer time selectivity was reduced.

Preliminary studies in our laboratory demonstrated that commercial BG agars were quite variable in selectivity. We therefore undertook an investigation of factors affecting the selectivity of BGA, the results of which are reported in this paper.

MATERIALS AND METHODS

Cultures. Strains of Salmonella anatum, S. derby, S. blockley, Escherichia coli, Enterobacter aerogenes, Proteus mirabilis, Shigella sonnei, Arizona sp., and Citrobacter sp. were selected from stock cultures maintained in our laboratory. Additional Arizona cultures were obtained from Alice Moran, Animal and Plant Health Inspection Service, U.S. Department of Agriculture. Three strains of bacteria isolated from commercial turkey noodle soup mix which showed luxuriant spreading growth on BGA were also included in the test series. These were identified on the basis of biochemical tests as described by Edwards and Ewing (2) as E. aerogenes.

Chemicals and growth media. Sources of chemi-
cals and media used were as follows: Trypticase soy broth (TSB), Trypticase soy agar (TSA), and bile salts (BBL); sodium dodecyl sulfate, lactose, sucrose, and phenol red (Fisher Scientific Co.); brilliant green (Matheson, Coleman, and Bell); sodium glycoaurachololate (Nutritional Biochemicals Corp.); sulfathiazole, sulfapyridine, and sulfadiazine (Pfaltz and Bauer); and sulfanilamide (Eastman Organic Chemicals). Other bacteriological media were from BBL or Difco. Commercial growth media were prepared according to the manufacturers' instructions.

Preparation of brilliant green agars. The basic formula for 1 liter of medium consisted of 40 g of TSA (dehydrated), 10 g of sucrose, 10 g of lactose, and 80 mg of phenol red. These were suspended in 1 liter of distilled water together with any optional ingredients included, such as sulfonamides, bile salts, etc., and the mixture was stirred until the powders were completely wetted, giving a uniform suspension. The pH was checked and adjusted to 6.7 if necessary. The suspension was then heated until it was completely dissolved and was then dispensed into containers of known volume prior to autoclaving at 121 C for 15 min. After sterilization, the medium was brick red. The sterilized agar was cooled to 50 C in a 50 C water bath. Then BG was added aseptically to the indicated concentration from a filter-sterilized stock solution containing 1.0 mg/mL, and the mixture was stirred and allowed to stand for 5 min. The agar then was poured into sterile petri dishes.

Determination of inhibition of BG agars. The test strains of bacteria were grown in TSB for 24 h at 37 C. They were streaked directly from TSB onto the agar plates. The plates were observed after incubation for 24 h at 37 C. For the spread plating experiments, 0.2 ml of 10^-4 and 10^-7 dilutions were spread on the agars by using a glass "hockey stick." The plates were prepared in duplicate. The controls were pour-plated by using Trypticase soy agar (BBL). Counts were made after 24 and 48 h of incubation at 37 C.

Spectrophotometric measurements. Spectrophotometric measurements were made by using a Beckman DPC double-beam spectrophotometer with a recorder. Cuvettes with a 1-cm light path were used. For measurements on agars, the liquid media were poured into the cuvettes and allowed to solidify.

RESULTS AND DISCUSSION

In preliminary experiments with media containing BG dye, we observed that the dye was markedly less inhibitory to bacteria when it was added to the medium prior to autoclaving than when it was added after the remainder of the medium was autoclaved. Autoclaving also partially decolorized the dye. These observations will be discussed in more detail in another paper. It is undoubtedly feasible to add excess dye and to adjust its inhibitory properties by a specified amount of heat treatment (4, 14). However, variations in the performance of the autoclave, geometry of the containers, etc., may easily alter the effective heat treatment which the media receive, which in turn will affect the inhibitory properties of media made by this procedure. It therefore appeared more reasonable to add dye after sterilization of the remainder of the medium as recommended by many authors (5, 9, 10), and we adopted this approach.

The inhibitory properties of experimental media were evaluated either by streaking or spread plating. Streaking provided a gradient in the concentration of cells from the beginning of the streak. Sometimes bacteria grew only at the beginning of the streak, indicating that high concentrations of cells overcame the inhibitory effects of the media. Streaking provided little information as to the proportion of cells which formed colonies unless there was little or no growth. The relative sizes of whatever colonies did form were compared, giving a measure of the degree of inhibition. Spread plating was used to learn what proportion of cells formed colonies on the media as compared with a noninhibitory control. This is more laborious than streaking, and dilution of stock cultures was required to obtain countable plates. Cells were well separated by this method so concentration effects should not be a factor, and this method therefore gave a more exacting comparison of inhibitory properties of media than streak plating.

Results obtained with BGA formulations with and without sulfanilamide (SN) and also the results with commercial BGS agar are shown in Table 1. With BG alone, concentrations which completely inhibit most of the other test organisms also significantly inhibit the salmonellae. Addition of SN substantially improved the selectivity. SN was particularly valuable in retarding growth of the Entrobacter isolates which, in its absence, showed a luxuriant spreading growth which obscured everything else on the plates. A commercial BGS agar did not significantly inhibit any of the test organisms.

Table 2 shows a comparison of four sulfonamides. SN was distinctly less inhibitory to salmonellae than the other three, and it was judged to have slightly better selective properties. Its use in BGA has not previously been reported.

Stokes and Osborne (16) added sodium glycoaurachololate to their brilliant green-selenite enrichment medium to counteract the toxicity of BG. Miller and Banwart (11) observed that bile salts reduced the toxicity of BG to a number of types of bacteria. Jameson (7) found that sodium dodecyl sulfate inhibited Proteus. Results when sodium glycoaurachololate, bile
salts, and sodium dodecyl sulfate were added to the BG medium are shown in Table 3. The toxicity of the medium to all the test strains, with the possible exception of Proteus and Shigella, was markedly reduced with all three substances, and the effects were similar. The selectivity of the medium was not improved over that with BG alone. Addition of sulfonamides (not shown) did not improve the selectivity in the presence of the bile salts.

Three experimental brilliant green-phenol red-sulfanilamide agars were compared with a number of commercial agars recommended for isolation of salmonellae using spread plating (Table 4). Four commercial BGA were included as well as bismuth sulfite (BSA), salmonella-shigella (SS), and Hektoen (8) agars.

The absorption spectra of the prepared commercial BG media (Fig. 1) showed that most of the dye present (12.5 mg/liter according to the manufacturer) in them was decolorized during preparation. The absorption at 630 nm is less
than 5 nm.

Arizona Proteus mirabilis............

S. Enterobacteraerogenes

Isolate

Enterobacter aerogenes

Hektoen, S. agars

mg/liter)-sulfadiazine

Escherichia coli.............

Enterobacter aerogenes

-5

S. mirabilis

Arizona sp.

Citrobacter sp.

Shigella sonnei

Enterobacter aerogenes

isolate

1

3

5

TABLE 3. Growth of bacteria in 24 h after streaking on BG agar containing bile salts and sodium dodecyl sulfate

| Bacteria                      | Growth of bacteria on BG agar containing (1 g/liter): |
|-------------------------------|------------------------------------------------------|
|                               | Sodium glycosaurocholate | Sodium dodecyl sulfate | Bile salts (BBL) |
|                               | 5a | 10 | 15 | 20 | 5  | 10 | 15 | 20 | 5  | 10 | 15 | 20 |
| Salmonella anatum.............. | + + | + | + | + | + | + | + | + | + | + | + | + |
| S. derby.............          | + + | + | + | + | + | + | + | + | + | + | + | + |
| S. blockley.................. | + + | + | + | + | + | + | + | + | + | + | + | + |
| Escherichia coli.............. | + + | + | + | + | + | + | + | + | + | + | + | + |
| Enterobacter aerogenes........| + + | + | + | + | + | + | + | + | + | + | + | + |
| Proteus mirabilis.............| + + | + | - | + | + | + | - | + | + | - | + | - |
| Arizona sp...................| + + | + | ± | + | + | + | - | + | + | - | + | - |
| Citrobacter sp..............  | + + | + | + | + | + | + | + | + | + | + | + | + |
| Shigella sonnei................| - | - | - | - | - | - | - | - | - | - | - | - |
| Enterobacter aerogenes isolates | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |

* BG agar concentration (mg/liter).

+ +. Normal growth; +, growth inhibited; ±, slight growth at beginning of streak; -, no growth.

TABLE 4. Comparison of recoveries of salmonellae and other Enterobacteriaceae on various agars

| Organism                       | Percent of counts on Trypticase soy agar* |
|--------------------------------|------------------------------------------|
|                               | BGA* | BGA-SP-1 | BGA-SP-2 | BGA-SD | BGA-SN 1-5 | BGA-SN 2-5 | BGA-SN 1-10 | Hektoen | BSA | SS |  |
|--------------------------------|------|----------|----------|--------|------------|------------|------------|--------|-----|----|---|
|                               | 246  | 48       | 24       | 48     | 24         | 48         | 24         | 48     | 24  | 48 | 24 |
| Salmonella blockley........... | 100  | 103      | 89       | 92     | 106        | 104        | 102        | 102    | 77  | 80 | 109 |
| S. oranienberg..............   | 115  | 119      | 118      | 120    | 132        | 122        | 119        | 118    | 127 | 127 | 117 |
| S. derby..............         | 115  | 110      | 107      | 99     | 97         | 111        | 101        | 107    | 89  | 97 | 89 |
| S. anatum.....................| 111  | 112      | 96       | 101    | 101        | 114        | 112        | 106    | 101 | 108 | 107 |
| Escherichia coli.............. | 116  | 112      | 63       | 92     | 120        | 107        | 104        | 109    | 0   | 0  | 0  |
| Proteus mirabilis.............| 82   | 79       | 0        | 76     | 78         | 86         | 99         | 86     | 0   | 0  | 0  |
| Citrobacter sp..............  | 91   | 93       | 0        | 63     | 0          | 88         | 96         | 99     | 0   | 0  | 0  |
| Arizona sp............        | 100  | 98       | 65       | 89     | 95         | 101        | 110        | 113    | 90  | 87 | 109 |
| Enterobacter aerogenes........| 84   | 82       | 87       | 89     | 87         | 95         | 91         | 87     | 71  | 86 | 67 |
| Isolate 1, E. aerogenes...... | 68   | 76       | 81       | 89     | 79         | 85         | 76         | 91     | 54  | 85 | 49 |

* Trypticase soy agar plates were pour plated; others were spread plated. All plates were made in duplicate.

* BGA, Brilliant green agar (12.5 mg of BG/liter) (Commercial); BGA-SP-1 and BGA-SP-2, brilliant green (12.5 mg/liter) + sulfapyridine (1.0 g/liter) from manufacturers 1 and 2; BGA-SD, brilliant green (12.5 mg/liter)-sulfadiazine (80 mg/liter) agar (Commercial); BGA-SN, experimental brilliant green-sulfanilamide agars 1-5 (1 g of SN, 5 mg of BG/liter), 1-10 (1 g of SN, 10 mg of BG/liter), 2-5 (2 g of SN, 5 mg of BG/liter); Hektoen, Hektoen agar; BSA, bismuth sulfite agar; SS, salmonella-shigella agar.

* Hours of incubation at 37 C.

* Uncountable because of smearing.

than that of the experimental media containing 5 mg/liter. There was no absorption peak at 630 nm for BGA.

Recoveries of salmonellae and Arizona were comparable to the TSA controls on all BG agars with the exception of the BGA-SN 1-10, where recoveries were somewhat lower (Table 4). Growth of salmonellae was retarded on the more selective BG agars, but characteristic pink colonies formed in 24 h on all but the BGA-SN 1-10 formulation. Salmonellae colonies were tiny and difficult to see after 24 h on BGA-SN 1-10,
and no Arizona could be detected. However, characteristic pink colonies of salmonellae and Arizona formed after 48 h of incubation. Of the other agars, recoveries of salmonellae and Arizona were good on BSA, but were lower on Hektoen and SS agars than on the BG agars. Some BSA plates were uncountable because of smearing, and their results are somewhat erratic.

The experimental BGA-SN showed superior selectivity to all the commercial agars. All three formulations totally suppressed growth of E. coli, Citrobacter, and P. mirabilis. The BGA-SN 1–10 formulation, in addition, totally suppressed growth of one E. aerogenes strain. On BGA-SN 2–5, the E. aerogenes strains formed tiny faintly pink colonies, in contrast to the green colonies formed on the other BGA-SN. These might be confused with salmonellae. The commercial BG agars showed little selectivity. One (BGA-SP-1) suppressed growth of Proteus. Hektoen agar was quite inhibitory to E. coli and Citrobacter, BSA retarded the growth of Enterobacter and suppressed the growth of Citrobacter, and SS agar prevented the growth of E. coli and Citrobacter.

On BG agars, growth of bacteria frequently was inhibited (as shown) by reduced colony size without any reduction in the number forming colonies.

On Hektoen and SS agars, on the other hand, the number of cells forming colonies was markedly lower than on TSA, but the colonies which did form were large.

Three BGA were tested further on a more extensive series of stock cultures to insure that our test series did not contain atypical strains (Table 5). Commercial BGA and BGA-SN 1–5 and 1–10 were included by using TSA as a control. The results confirm the excellent selectivity of BGA-SN 1–5. All strains of salmonellae tested grew on it. The only interfering organisms which also grew on it were Klebsiella, E. aerogenes, and Pseudomonas aeruginosa. BGA supported excellent growth of Salmonella but also supported growth of many other types of bacteria. The BGA-SN 1–10 formulation appears excessively toxic. BGA-SN 1–5 supported growth of only one of four Arizona strains, whereas three of four grew on BGA. BGA-SN 1–5 appears potentially useful for the isolation of specific bacteria.

### Table 5. Inhibition of bacteria of BG agars

| Bacteria                     | No. of strains tested | No. of strains forming colonies on: |
|------------------------------|-----------------------|------------------------------------|
|                              | BGA (%)               | BGA-SN 1–5 (%)                     | BGA-SN 1–10 (%)                  |
| Escherichia coli             | 10                    | 8                                  | 0                                 |
| Enterobacter aerogenes       | 5                      | 5                                  | 3                                 |
| E. hafnia                    | 2                      | 1                                  | 0                                 |
| E. cloacae                   | 1                      | 0                                  | 0                                 |
| Klebsiella sp.               | 1                      | 1                                  | 1                                 |
| K. pneumoniae               | 1                      | 1                                  | 1                                 |
| Proteus vulgaris             | 1                      | 0                                  | 0                                 |
| P. mirabilis                 | 6                      | 3                                  | 0                                 |
| P. rettgeri                  | 1                      | 0                                  | 0                                 |
| Providencia sp.              | 1                      | 0                                  | 0                                 |
| Alcaligenes faecalis         | 2                      | 1                                  | 0                                 |
| Arizona sp.                  | 4                      | 3                                  | 1                                 |
| Citrobacter sp.              | 1                      | 1                                  | 0                                 |
| Shigella sonnei              | 2                      | 2                                  | 0                                 |
| Pseudomonas sp.              | 1                      | 1                                  | 0                                 |
| Pseudomonas aeruginosa       | 1                      | 1                                  | 1                                 |
| Serratia marcescens          | 1                      | 0                                  | 0                                 |
| Streptococcus pyogenes       | 1                      | 0                                  | 0                                 |
| S. faecalis                  | 5                      | 0                                  | 0                                 |
| Staphylococcus aureus        | 3                      | 0                                  | 0                                 |
| Bacillus cereus              | 24                     | 24                                 | 24                                |
| Salmonella enteritidis ser.  | 24                    | 24                                 | 18                                |
| S. pullorum                  | 1                      | 1                                  | 0                                 |
| S. gallinarum                | 1                      | 1                                  | 0                                 |

*Abbreviations are as in Table 4.*
of bacteria of the *E. aerogenes-Klebsiella* group. All strains of these organisms tested grew on BGA-SN, and none of the other bacteria tested formed green colonies on this medium.

The BGA-SN formulations are particularly advantageous in that they totally prevent growth of *Proteus* sp. which mimic *Salmonella* and *Arizona* on all the other agars tested. The only organisms forming pink colonies on BGA-SN were *Pseudomonas* sp., and these do not so closely resemble *Salmonella* colonies.

An ideal selective and differential medium for *Salmonella* would permit all cells of *Salmonella* to form colonies of characteristic appearance. Growth of other organisms should be suppressed, or, if they do grow, they should form colonies different in appearance from salmonel- lae and should not mask *Salmonella* colonies. No one agar tested was ideal in all respects. The BGA-SN 1–5 formulation most nearly met the above criteria. Its main drawback was that some strains of *Arizona* did not grow on it.

From the data given in this paper it is possible to easily and reproducibly prepare BG agars of any desired inhibitory properties. For isolation of particularly sensitive strains of *Salmonella* or *Arizona* or where rapid growth is desired and few interfering bacteria are expected, a less inhibitory formulation may be advantageous. A more inhibitory formulation may be advantageous when large numbers of other bacteria are present.

Since our results indicate that all components of the media but the BG dye are reasonably stable, it should be possible to prepare commercially one or more formulations of the phenol red-sulfonamide agar base. These could be made up and sterilized in the usual manner with instructions to the preparer to add BG dye to the desired concentration as we have described after sterilization. By using this approach, it should be possible to prepare brilliant green-phenol red agars with the same reproducibility and selectivity as we have reported, using a commercially available base.

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