Inhibition of Injured *Escherichia coli* by Several Selective Agents

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Received for publication 27 August 1970

A population of *Escherichia coli* ML30 cells was exposed to a quaternary ammonium compound, and injury to the cells was measured by a comparison of counts on Trypticase Soy Agar and Violet Red Bile Agar. Substantial injury could not be detected with a minimal medium. The ingredients of Violet Red Bile Agar were tested against damaged cells. The bile salts mixture alone in the medium prevented as many injured cells from growing as did any combination of the selective agents and inhibited as many injured bacteria as were inhibited by Violet Red Bile Agar itself. These dyes and salts were similarly assayed in minimal agar, and comparable results were obtained. Individual bile salts and other potential selective agents were added to the minimal medium, and the media were tested for inhibition of injured *E. coli*. Sodium deoxycholate was the bile salt most inhibitory to damaged *E. coli* cells.

The detection of pathogens, indicator, or spoilage bacteria should be accomplished regardless of the vigor of the cells being sought. Increasing evidence indicates that currently used selective media often fail to detect all of the desired microorganisms. In an earlier study, we reported on sanitizer-induced injury of coliform and other bacteria and the expression of this injury on selective media (4). Coliform organisms are still used widely in assessing the sanitary quality of foods and water. These products and their environments that are examined for these bacteria often have been subjected to treatments that may cause sublethal damage of coliforms. Since selective media are often unable to detect injured coliforms, methods are needed that would overcome this deficiency.

The objective of this study was to identify the specific agents in selective media that impair the growth of injured *Escherichia coli*. This knowledge would be useful in designing new and improved selective media.

**MATERIALS AND METHODS**

**General procedures.** The culture, its preparation and exposure to sanitizer, the methylalkylbenzyltrimethyl ammonium chloride (a quaternary ammonium compound (QAC)) neutralization of sanitizer, and plating methods were as previously described (4).

**Media.** Trypticase Soy Broth (TSB), Trypticase Soy Agar (TSA), Violet Red Bile Agar (VRB), and Deoxycholate Agar were obtained in the dehydrated form (BBL). A basal medium without the VRB selective agents was prepared containing, per liter: yeast extract (BBL), 3.0 g; Gelysate (BBL), 7.0 g; lactose (Difco), 10.0 g; and agar (Difco), 15.0 g. In some cases, 5.0 g of NaCl (Fisher Scientific Co., Pittsburgh, Pa.) was added to the above basal medium before autoclaving. The basal medium was dispensed in 97-ml quantities to which 1.0 ml of selective agents were added just prior to pouring plates. Lauryl Sulfate Agar (LSA) was prepared containing, per liter: Trypticase (BBL), 20.0 g; lactose (BBL), 5.0 g; K$_2$HPO$_4$ (Baker Chemical Co.), 2.75 g; KH$_2$PO$_4$ (Fisher), 2.75 g; NaCl (Fisher), 5.0 g; sodium lauryl sulfate (Sigma Chemical Co., St. Louis, Mo.), 0.1 g; agar (Difco), 15.0 g. The media were dispensed in about 100-ml quantities and autoclaved for 15 min at 121°C (except the VRB which as directed was not autoclaved). Media not used immediately were stored at room temperature, melted by placing in flowing steam for 25 min, and tempered to 46°C prior to use. A minimal agar (MA) was prepared as previously described (4).

**Selective agents.** Stock solutions of dyes and salts were prepared in distilled water at concentrations 100-fold greater than normally present in the selective media and were sterilized at 121°C for 15 min. This permitted the addition of 1.0-ml quantities to 99 ml of medium for the desired concentration of selective agent. The dyes and their stock concentration in preparation were, per 100 ml: crystal violet (Allied Chemicals), 0.02 g; neutral red (Fisher), 0.3 g; eosin Y.
deoxycholate; laurylsulfate were stored distilled water at 121°C until 25% of the cells surviving treatment but also inhibited about 25% of the untreated cells. Crystal violet in the basal medium with NaCl did not inhibit the untreated cells, but it inhibited more of the treated cells than did crystal violet in MA. With crystal violet in either medium, the plates had to be incubated for about...

RESULTS

Effectiveness of various plating media for recovery of injured bacteria. The number of bacteria which grew on various plating media depended on the type of medium, on the treatment given to the bacteria, and on their condition before plating. Ideally, selective media should support equivalent growth and result in counts of untreated cells equal to nonselective media. With some of the individual media this did not occur and was taken into account in the calculation of injury as in a previous report (4). Near the end of the lag and the early logarithmic phases of growth, untreated cells of E. coli ML30 produced markedly fewer colonies on the VRB and MA media (Fig. 1). The counts thereafter on MA more closely approached those of TSA, whereas counts on VRB continued to be lower until the physiological growth age of the culture, subsequent experiments on treatments with QAC were done by using cultures grown for 12 hr at 35°C in TSB.

Of the selective media tested (Fig 2), LSA inhibited the fewest sanitizer-treated bacteria. Violet Red Bile Agar was intermediate and Desoxycholate Agar inhibited the largest percentage of bacteria.

Inhibitory properties of various dyes. Eosin Y added to MA at the concentration used in Eosin Methylene Blue Agar (1) had no apparent effect on E. coli before or after treatment with QAC (Table 1). Brilliant Green at the concentration used in Brilliant Green Agar (1) inhibited growth of E. coli ML30 before and after sanitizer treatment. Neutral red at the concentration used in VRB inhibited some treated cells but had little effect on the untreated cells. When the same concentration of neutral red was added to the basal medium with NaCl, a greater number of treated cells was inhibited. Crystal violet added to MA at the concentration used in VRB inhibited nearly half of the cells surviving treatment but also inhibited about 25% of the untreated cells. Crystal violet in the basal medium with NaCl did not inhibit the untreated cells, but it inhibited more of the treated cells than did crystal violet in MA. With crystal violet in either medium, the plates had to be incubated for about...

Fig. 1. Colony counts on different media of Escherichia coli ML30 growing in Trypticase Soy Broth at 35°C.

Fig. 2. Growth of QAC-treated and untreated cells of Escherichia coli ML30 in several plating media.
TABLE 1. Effect of dyes in plating media on the colony count of Escherichia coli ML30

| Medium          | Additive       | Per cent of population<sup>a</sup> which did not grow |
|-----------------|----------------|-------------------------------------------------------|
|                 |                | Un-treated cells | Treated<sup>b</sup> cells                  |
| Trypticase Soy Agar | None           | 0                | 10                                      |
| Violet Red Bile Agar | None           | 5                | 87                                      |
| Minimal agar    | None           | 3                | 7                                       |
| Minimal agar    | Brilliant Green| 99.9             | 99.9                                    |
| Minimal agar    | Neutral red    | 5                | 20                                      |
| Minimal agar    | Crystal violet | 25               | 48                                      |
| Minimal agar    | Crystal violet | 35               | 37                                      |
| Basal NaCl      |                | 0                | 33                                      |
| Basal NaCl + neutral red | 0     | 38                      |
| Basal NaCl + crystal violet | 0 | 57                      |
| Basal NaCl + crystal violet + neutral red | 0 | 54                      |

<sup>a</sup> About 10<sup>8</sup> cells per ml.
<sup>b</sup> Treatment with 30 μg of QAC per ml (product A) for 60 sec at 0 C.

TABLE 2. Effect of selective agents in plating media on the colony count of Escherichia coli ML30

| Medium          | Additive                      | Per cent of population<sup>a</sup> which did not grow |
|-----------------|-------------------------------|-------------------------------------------------------|
|                 |                               | Un-treated cells | Treated<sup>b</sup> cells                  |
| Trypticase Soy Agar | None            | 0                | 5                                       |
| Minimal agar    | None             | 3                | 4                                       |
| Minimal agar    | Bile salts mixture      | 11               | 65                                      |
| Violet Red Bile Agar | None           | 0                | 62                                      |
| Basal NaCl      |                               | 0                | 23                                      |
| Basal NaCl      | Bile salts mixture      | 0                | 80                                      |
| Basal NaCl      | Bile salts mixture + crystal violet + neutral red | 0 | 75                                      |
| Basal NaCl      | NaCl + bile salts mixture | 0                | 33                                      |
| Basal NaCl      | NaCl + bile salts mixture + crystal violet + neutral red | 37 | 98                                      |

<sup>a</sup> About 10<sup>8</sup> cells per ml.
<sup>b</sup> Treatment with 30 μg/ml of QAC (product A) for 60 sec at 0 C.

72 hr to allow for the formation of colonies large enough to be counted easily. Incubation beyond 72 hr did not increase the counts. Crystal violet and neutral red in combination did not appear to be more toxic than the crystal violet alone. No medium that contained a dye and allowed growth inhibited as many treated cells as did VRB.

Inhibitory properties of various salts. When a bile salts mixture similar to that in VRB was added to MA, the count of untreated cells was reduced slightly. This medium inhibited a majority of cells surviving treatment with the QAC (Table 2). Counts on MA were essentially the same as on TSA. MA with the bile salts mixture inhibited nearly the same number of treated cells as did the VRB. Counts on the basal medium containing the bile salts mixture were similar to those obtained on VRB. The basal medium containing both NaCl and the bile salts mixture inhibited many untreated cells and inhibited more treated cells than did VRB. The addition of neutral red and crystal violet to the basal medium (with or without NaCl) containing the bile salts mixture did not significantly affect the counts.

Bile salts added separately to MA influenced the colony count differently (Table 3). The bile salts mixture in MA inhibited 65% of the cells surviving treatment. Sodium deoxycholate in MA inhibited many of the treated cells and inhibited 46% of the untreated cells. Sodium

TABLE 3. Effect of various bile salts in the plating medium on the colony count of Escherichia coli ML30

| Medium          | Additive                      | Per cent of population<sup>a</sup> which did not grow |
|-----------------|-------------------------------|-------------------------------------------------------|
|                 |                               | Un-treated cells | Treated<sup>b</sup> cells                  |
| Trypticase Soy Agar | None            | 0                | 5                                       |
| Minimal agar    | None             | 0                | 10                                      |
| Minimal agar    | Bile salts mixture      | 20               | 65                                      |
| Minimal agar    | Cholic acid          | 0                | 0                                       |
| Minimal agar    | Sodium deoxy- cholate     | 46               | 93.3                                    |
| Minimal agar    | Lithocholic acid       | 0                | 0                                       |
| Minimal agar    | Sodium glyco- cholate     | 7                | 41                                      |
| Minimal agar    | Taurocholic acid        | 0                | 7                                       |
| Minimal agar    | Cholesterol            | 0                | 0                                       |
| Minimal agar    | Sodium lauryl sulfate    | 0                | 19                                      |

<sup>a</sup> About 10<sup>8</sup> cells per ml.
<sup>b</sup> Treatment with 30 μg of QAC per ml (product A) for 60 sec at 0 C.
glycocholate in MA inhibited very few untreated cells and inhibited fewer treated cells than the MA containing the bile salts mixture. The incorporation of cholic acid, taurocholic acid, lithocholic acid, or cholesterol separately into MA resulted in counts of treated and untreated cells that were essentially the same as the TSA counts. The addition of sodium lauryl sulfate to MA in the concentration used in LSA resulted in no inhibition of untreated cells and limited inhibition of treated cells.

**DISCUSSION**

The cells treated with the QAC grew essentially equally on the complete and minimal media, indicating that the treatment had not caused metabolic injury. Growth of the stressed or treated cells was markedly reduced on Desoxycholate Agar and VRB, whereas on LSA their growth was less affected. LSA is infrequently used even though it is less inhibitory to treated cells because it is not sufficiently selective for coliforms (2). Detection of QAC stressed coliforms would therefore be expected to vary depending on the medium selected. Desoxycholate Agar inhibited both treated and untreated cells. In view of such toxic properties for unstressed cells, this medium was not selected for further study. Violet Red Bile Agar supported growth of all viable cells of *E. coli* ML30 before sanitizer exposure but inhibited some cells that survived the QAC treatment. The basal medium (i.e., VRB without selective agents or NaCl) supported growth of the cells; this permitted an evaluation of the action of the selective agents in VRB on stressed cells. Therefore, this medium was chosen for further study of injury and inhibition.

Of the dyes commonly used as selective for coliforms, Brilliant Green was found to be inhibitory to untreated as well as treated cells. Thus, a medium containing Brilliant Green would not appear to be a good medium for enumeration of all viable *E. coli*. Eosin Y, which is usually used in differential media and not in selective media, was not inhibitory, as might be expected. Of the dyes which are components of VRB, crystal violet was more inhibitory than neutral red, but the combination was not more inhibitory than crystal violet alone. In any case, the amount of inhibition was not as great as with VRB; therefore, the dyes alone were not the only agents in VRB which inhibited injured cells.

The other selective agent in VRB was the bile salts mixture which, when added to the basal medium, revealed more injured cells than did VRB. Since crystal violet and neutral red added with the bile salts mixture showed no increase in observed injury, it was concluded that the bile salts mixture inhibited damaged bacteria that were susceptible to inhibition by the dyes plus others not affected by the dyes.

Sodium deoxycholate was the most inhibitory of the bile salts in the bile mixture, and this agrees with the results obtained with Desoxycholate Agar. The reason for the uniquely high inhibitory activity of sodium deoxycholate was not readily apparent. Recently, Hill (3) has shown that bile salts dissolve part of the cell wall material from disrupted cells of *E. coli*. In the study by Hill, sodium deoxycholate was the most active bile salt, and sodium lauryl sulfate was also active. This same action may have occurred with cells injured by the QAC. Sodium glycocholate inhibited some injured cells but not as many as VRB. The other bile salts tested inhibited very few, if any, injured cells. Since cholesterol is structurally related to the bile salts, its inhibitory activity against damaged cells was tested but found to be negligible.

The action of QAC on the cells of *E. coli* is not understood. Regardless of the site of damage, the QAC made the cell more sensitive to the selective agents in certain media. The action of the selective agent on sanitizer-injured cells has to be dependent upon the preliminary action of the sanitizer, otherwise injury under these conditions could not be observed. Regardless of the mode of action of the QAC and selective agents, some injured bacteria were not able to grow on the selective media, and this decrease in count may be significant in the public health acceptance or rejection of foods.

**ACKNOWLEDGMENTS**

This investigation was supported by Public Health Service training grant ES-61 from the Division of Environmental Health Sciences, and by Public Health Service research grant no. FD 00085 from the Food and Drug Administration.

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