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CHAPTER 10

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Comparison of Media for the Radiometric Detection  
of Anaerobic Spores

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An inoculum of heat-shocked spores from putrefactive anaerobe (PA) 3679 ( $10^5$  spores per ml of Tryptic Soy Broth), rendered anaerobic with nitrogen gas and containing  $0.0139 \mu\text{Ci}$  of  $^{14}\text{C}$  glucose/ml, did not allow sufficient  $^{14}\text{CO}_2$  to be produced within 8 hr to ascertain the presence of viable bacteria by radiometric methods. Therefore, this medium was modified in a variety of ways in order to enhance its ability to support the growth of anaerobic spores with concomitant production of  $^{14}\text{CO}_2$  from labeled substrate. The best medium tested was Tryptic Soy Broth supplemented with  $^{14}\text{C}$ -labeled substrate, thiotone, yeast extract, sodium bicarbonate, and sodium thioglycollate. Inocula of  $10^2$  to  $10^7$  heat-shocked spores of PA 3679 or *Clostridium botulinum* strain 62A, per milliliter of medium, were detected within 11 to 1 hr, respectively. When  $10^5$  or fewer *Salmonella typhimurium* or *Staphylococcus aureus* cells were present per milliliter of this medium, detection times were about 3-4 hr faster than those of comparable numbers of spores. A mixture of sterile beef loaf with any of the species tested did not interfere with detection in this medium.

INTRODUCTION

The detection of aerobic bacteria by the evolution of  $^{14}\text{CO}_2$  from labeled glucose has been demonstrated by several laboratories (DeBlanc et al., 1971, DeLand and Wagner, 1969, 1970; Waters, 1972). However, this work has centered on clinical specimens and no methods have been described for the detection of anaerobic spores. This paper summarizes some of the work on media tested in our laboratories for detection of spores from a putrefactive anaerobe (PA) 3679, *Clostridium botulinum* strain 62A, *Salmonella typhimurium* strain RIA, and *Staphylococcus aureus* var. Giorgio. Experiments with PA 3679 spores (*Clostridium sporogenes*) are described as a model to illustrate the comparative effects of different media on detection of anaerobic spores. This species was chosen as a representative of food spoilage anaerobes. Its behavior in the detection system described is similar to that of *C. botulinum*, strain 62A (Previte, unpubl. data).

METHODS AND MATERIALS

Spores of PA 3679 (*Clostridium sporogenes*) were prepared in biphasic culture (Anellis and Rowley, 1970). They were stored in replicate aliquots at  $-25 \text{ C}$ , thawed, heat-shocked at  $80 \text{ C}$  for 10 min, then diluted and inoculated into a variety of media used

for radiometric detection (Table 1). Colony counts were made in 12 x 208 mm tubes containing Thiotone Yeast Extract Agar (TYETA) which contained 0.5% Trypticase, 0.5% Yeast Extract, 5% Thiotone, 0.05% Sodium Thioglycollate, and 0.75% purified agar. It was maintained at 45-50 C until added to a 1-ml dilution of spores plus 0.2 ml of filter-sterilized 5% NaHCO<sub>3</sub>. The mixture was cooled, solidified, and incubated at 37 C for 24-48 hr.

Serial dilutions of the heat-shocked spores were inoculated with syringes into vaccine-capped vials containing the different media described in Table 1. The source of all media was Baltimore Biological Laboratories (Cockeysville, Md.). Generally, uniformly labeled <sup>14</sup>C glucose containing 3-3.5 mCi/0.18 g was in Tryptic Soy Broth as supplied by Johnston Laboratories (Cockeysville, Md.). In those vials containing 0.2500 μCi/ml or more, the activity of the glucose was 5.0 mCi/0.18 g (New England Nuclear Corp., Boston, Mass.). In all cases the labeled glucose was added prior to autoclaving the media.

The basic detection medium consisted of Tryptic Soy Broth (TSB) without glucose (column 2, Table 1) or the same medium supplemented with Yeast Extract (TSBYE, column 3, Table 1) and Thiotone plus different levels of <sup>14</sup>C substrate (TSS, Columns 4, 6, 7, 8, Table 1). L-alanine has been reported to enhance the germination of *C. botulinum* spores (Rowley and McCall, 1972). Since the amount of this amino acid in Thiotone or Yeast Extract has not been reported, it was added to TSS to determine whether it would improve detection time (TSSA, column 8, Table 1). The addition of NaHCO<sub>3</sub> also has been reported to improve germination of some spore types (Rowley and Feeherry, 1970). Therefore, 0.023 ml of filter-sterilized 5% NaHCO<sub>3</sub> per ml was added to all media excepting TSB (column 2, Table 1). It was thought that a higher ratio of labeled to nonlabeled carbohydrate might improve detection time. TSB contains phytone which has

TABLE 1. Composition of detection media

| Constituents of Media                         | (2)            | (3)            | (4)            | (5)   | (6)            | (7)   | (8)   | (9)   |
|---|----------------|----------------|----------------|---|----------------|---|---|---|
|   | TSB            | TSBYE          | TSS            | TSB Pre-reduced <sup>a</sup>                    | TSS            | TSS   | TSSA  | PPM   |
| μCi/ml <sup>14</sup> C Substrate <sup>d</sup> | 0.0139         | 0.0139         | 0.0139         | 0.0484 <sup>b</sup>                             | 0.0833         | 0.2500 <sup>c</sup>                             | 0.2500 <sup>c</sup>                             | 0.3280 <sup>c</sup>                             |
| Trypticase, 1.42%                             | +              | +              | +              | +   | +              | +   | +   | 0.085%  |
| Phytone, 0.25%                                | +              | +              | +              | +   | +              | +   | +   | —   |
| NaCl, 0.42%                                   | +              | +              | +              | +   | +              | +   | +   | 0.043%  |
| K <sub>2</sub> HPO <sub>4</sub> , 0.21%       | +              | +              | +              | +   | +              | +   | +   | 0.021%  |
| Thiotone, 3.91%                               | —              | —              | +              | —   | +              | +   | +   | 0.085%  |
| Sodium Thioglycollate 0.042%                  | —              | —              | +              | —   | +              | +   | +   | 0.042%  |
| Yeast Extract, 0.42%                          | —              | +              | +              | + <sup>a</sup>                                  | +              | +   | +   | —   |
| L-alanine, 0.071%                             | —              | —              | —              | —   | —              | —   | +   | 0.061%  |
| L-cysteine                                    | —              | —              | —              | + <sup>a</sup>                                  | —              | —   | —   | 0.012%  |
| Gas flush prior to incubation                 | N <sub>2</sub> | N <sub>2</sub> | N <sub>2</sub> | N <sub>2</sub> /CO <sub>2</sub> /H <sub>2</sub> | N <sub>2</sub> | N <sub>2</sub>                                  | N <sub>2</sub>                                  | N <sub>2</sub> /CO <sub>2</sub> /H <sub>2</sub> |
| Gas flush after sampling                      | N <sub>2</sub> | N <sub>2</sub> | N <sub>2</sub> | N <sub>2</sub> /CO <sub>2</sub> /H <sub>2</sub> | N <sub>2</sub> | N <sub>2</sub> /CO <sub>2</sub> /H <sub>2</sub> | N <sub>2</sub> /CO <sub>2</sub> /H <sub>2</sub> | N <sub>2</sub> /CO <sub>2</sub> /H <sub>2</sub> |
| Total volume in vial (ml)                     | 36             | 36             | 36             | 31  | 12             | 12  | 12  | 9.3   |

<sup>a</sup>The medium contains yeast extract, vitamin K, L-cysteine, and sodium polyanethol sulfonate. It stabilizes at an Eh of -150 mv as formulated by Johnston Laboratories, Inc.

<sup>b</sup>50% of label supplied as proprietary <sup>14</sup>C substrate (amino acid) by Johnston Laboratories, Inc.

<sup>c</sup>25% of label supplied as proprietary <sup>14</sup>C substrate (amino acid) by Johnston Laboratories, Inc.

<sup>d</sup>Glucose unless noted otherwise.

+ = constituent added to medium.

— = constituent not added to medium.

TABLE 2. Detection of PA 3679 spores in different media<sup>a</sup>

| Media            | $\mu\text{Ci/ml}$ of $^{14}\text{C}$ Glucose | Detection Time in Hours <sup>a</sup> |
|------------------|--|--------------------------------------|
| TSB              | 0.0139                                       | — <sup>b</sup>                       |
| TSBYE            | 0.0139                                       | — <sup>c</sup>                       |
| TSS              | 0.0139                                       | — <sup>c</sup>                       |
| TSB, Pre-reduced | 0.0484                                       | 10.0                                 |
| TSS              | 0.0833                                       | 7.0                                  |
| TSS              | 0.2500                                       | 6.5                                  |
| TSSA             | 0.2500                                       | 6.5                                  |
| PPM              | 0.3280                                       | 10.5                                 |

<sup>a</sup>The times shown are for detection of  $10^4$  spores/ml of medium unless noted otherwise.

<sup>b</sup>Negative within 8 hr after inoculation with  $10^5$  spores/ml.

<sup>c</sup>Negative within 12 hr of incubation.

37% carbohydrate (Baltimore Biological Laboratories, unpubl. data), therefore, a medium low in unlabeled carbohydrate was utilized, namely, PPM (Column 9, Table 1).

In the various media tested, the amount of label was varied, as was the volume of medium and the type of gas used, to attain anaerobiosis. Some vials were flushed for 1-2 min with nitrogen, at 10 lb./in<sup>2</sup> bubbled through the media, after filtration through a sterile, cotton-plugged syringe. A second syringe allowed excess gas to escape from the vial. Other sets of vials were flushed with a mixture of 85% N<sub>2</sub>, 10% CO<sub>2</sub>, and 5% H<sub>2</sub> delivered at 0.2 lb./in<sup>2</sup> through an anaerobic culture gas adapter (No. 225-025) attached to the Bactec Model 301 which was used to detect <sup>14</sup>CO<sub>2</sub> (Johnston Laboratories, Inc., Cockeysville, Md.). The contents of the vials were incubated at 37 C on a magnetic stirrer or G-2 rotary mixer at 275 rpm (New Brunswick Scientific Co., New Brunswick, N.J.). Both methods provided similar results.

Radioactive carbon dioxide was produced by spores which had, in most cases, germinated, emerged, elongated, and proceeded to vegetative cell metabolism and division. The presence of viable bacteria was considered positive when the labeled gas evolved from utilization of the <sup>14</sup>C substrate in the media provided a reading of at least 20% of scale on the Bactec Model 301. Readings were recorded every 30-60 min from the two vials containing the highest level of inocula until all the vials in a series became positive. The number of bacteria inoculated per milliliter of medium was plotted semi-logarithmically against detection time in hours for a series of dilutions of the original culture. The data provided a straight line. Detection times resulting from an inoculum of  $10^4$  spores/ml of medium were read from the graph and recorded in Table 2.

## RESULTS AND DISCUSSION

The composition of each medium tested is described in Table 1 and the detection times for an inoculum of  $10^4$ - $10^5$  PA 3679 heat-shocked spores/ml are listed in Table 2. When  $10^5$  spores were inoculated/ml of TSB containing 0.0139  $\mu\text{Ci}$  <sup>14</sup>C glucose/ml, detection was not attained within an 8-hr period (Table 2). Similarly,  $10^4$  spores/ml were not detected within 12 hr when the broth was supplemented with yeast extract (TSBYE) or with yeast extract and thiotone (TSS). When pre-reduced TSB containing 0.0484  $\mu\text{Ci}$  <sup>14</sup>C substrate/ml was used, spores were detected within 10 hr. Similar times were attained

with PPM. However, early detection times of 6.5-7 hr were attained with TSS or TSSA containing 0.0833  $\mu\text{Ci}$  or more of labeled substrate/ml of medium.

The use of  $\text{N}_2$ , compared to a  $\text{N}_2/\text{CO}_2/\text{H}_2$  mixture to fill the headspace in the vial after sampling, did not seem to affect detection time while the level of label seems to affect it significantly. None of the media with 0.0139  $\mu\text{Ci}$   $^{14}\text{C}$  substrate/ml allowed detection of  $10^4$ - $10^5$  spores within an 8-12-hr period while a level of 0.0484  $\mu\text{Ci}/\text{ml}$  or more did. An increase above 0.0833  $\mu\text{Ci}$   $^{14}\text{C}$  glucose/ml did not improve detection time significantly. When  $10^2$  to  $10^7$  PA 3679 spores were inoculated/ml of TSS containing 0.0833  $\mu\text{Ci}$   $^{14}\text{C}$  glucose/ml, detection was accomplished in 11 to 1 hr, respectively. Detection times of heat-shocked spores of *C. botulinum*, strain 62A, were comparable to those of PA 3679 while  $10^5$  or fewer *S. typhimurium* RIA or *S. aureus* var. Giorgio per ml of TSS were detected 3-4 hr faster than comparable numbers of spores. This difference corresponds to the time required for spores to germinate and proceed to the vegetative cell stage in a thiotone medium (Rowley et al., 1970). Detection times were comparable to those established for broth cultures when any one of the four organisms was blended into a sterile beef loaf at a level of  $10^4$  to  $10^6$  spores/g and placed as a 3-ml aliquot into TSS containing 0.0833  $\mu\text{Ci}$   $^{14}\text{C}$  glucose/ml.

The failure of some investigators (Washington and Yu, 1971) to attain positive results with certain bacterial species may be due, in part, to a lack of sufficient label or lack of utilization of the label provided. Both the level of  $^{14}\text{C}$  substrate as well as enrichment of the media appear to be critical for detection. The relatively poor performance of PPM (Table 2) might be due to the presence of too little total carbohydrate to allow rapid vegetative growth; that of pre-reduced TSB might be due to a lack of Thiotone enrichment.

Alanine was added to TSS since the amount of this amino acid contained in the ingredients of this medium has not been reported. Although it may enhance progression of spores to the vegetative stage in a less complex medium, its addition to TSS did not produce any noticeable effect on detection time under the experimental conditions described (Tables 1 and 2).

The results indicate that the best medium tested to date for detection of PA 3679 spores is TSS containing 0.0833 or 0.2500  $\mu\text{Ci}$  of  $^{14}\text{C}$  labeled substrate/ml. A mixture of  $^{14}\text{C}$  substrates in the media did not seem to enhance detection significantly compared to those containing only  $^{14}\text{C}$  glucose (columns 5, 7, 8, 9 versus 6, Table 1). However, the use of other labeled substrates in addition to  $^{14}\text{C}$  glucose might afford a more universal detection capability in that some microorganisms may convert little or no labeled glucose to  $^{14}\text{CO}_2$ .

The data accumulated in these preliminary studies demonstrate that the radiometric detection of bacteria in foods offers a promising potential. Future studies should focus on testing a variety of food-borne microorganisms in a model system as well as on improving the detection media, if it is necessary to do so. Finally, a series of studies involving inoculated packs of specific food types as well as specific foods with a natural flora would allow a determination of the suitability of the detection technique in different food systems.

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