

Radiometric Detection of Some Food-Borne Bacteria

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Studies on detection of bacteria by radiometric techniques have been concerned primarily with aerobic species in clinical specimens. The data presented here are related to detection of aerobic and anaerobic species that are of significance in foods, by measurement of $^{14}\text{CO}_2$ evolved from the metabolism of ^{14}C -glucose. *Salmonella typhimurium* and *Staphylococcus aureus* were inoculated into tryptic soy broth containing 0.0139 μCi of ^{14}C glucose/ml of medium. Detection times ranged from 10 to 3 hr for inocula of 10^9 to 10^4 cells/ml of broth. Heat-shocked spores of *Clostridium sporogenes* or *C. botulinum* were incubated in tryptic soy broth supplemented with Thiotone and NaHCO_3 . The medium was rendered anaerobic with N_2 . Spores were detected when 0.0833 μCi of labeled glucose was available/ml of medium but not when 0.0139 μCi of glucose was present/ml. The spores required 3 to 4 hr longer for detection than did comparable numbers of aerobic vegetative cells. The results demonstrate the importance of availability of sufficient label in the media and the potential of the application of this technique for sterility testing of foods.

Methodology classically used for bacterial detection requires a period of about 1 day up to weeks to certify the presence or absence of bacteria in canned foods (9). Recent publications have demonstrated detection of bacteria in blood or urine samples within a matter of a few hours. The method depends on evolution of $^{14}\text{CO}_2$ from metabolism of ^{14}C -glucose by bacteria (2-4, 11). Data have been accumulated primarily on aerobic organisms encountered in blood or urine samples. Little evidence is available concerning detection of anaerobes by this technique or on its application to the detection of bacteria in foods. This paper presents the results of preliminary work to establish methods for determining the potential application of radiometric techniques to the detection of bacteria in some foods.

MATERIALS AND METHODS

Cultures. A lyophilized culture of *Staphylococcus aureus* strain Giorgio was rehydrated, inoculated into tryptic soy broth (BBL), and incubated for 20 to 24 hr at 37 C. Serial dilutions were made in sterile distilled water, and the number of viable cells was determined from colony counts of streak plates made on tryptic soy agar.

Salmonella typhimurium strain RIA was grown and counted as above.

Putrefactive anaerobe 3679 (*Clostridium sporogenes*) was chosen as a representative food spoilage anaerobic sporeformer. It is encountered at times as a survivor in thermally underprocessed foods. Spores were prepared in a biphasic culture (1). They were stored at -25 C in 2-ml portions.

A tube was thawed and heated for 10 min at 80 C to kill the vegetative cells prior to each experiment. Serial dilutions were made in 1-ml amounts in sterile distilled water. They were added to tubes (12 by 208 mm) containing 0.2 ml of 5% filter-sterilized NaHCO_3 . A 10-ml amount of molten Thiotone yeast extract agar (TYETA) held at 45 to 50 C was added to each tube, and the tube contents were allowed to cool and solidify. This medium contained 0.5% Trypticase, 0.5% yeast extract, 5% Thiotone, 0.05% sodium thioglycolate, and 0.75% purified agar. It was adjusted to pH 7.2 with 5 N KOH prior to autoclaving. Average colony counts of triplicate tubes incubated at 37 C for 24 hr or more were used to establish that the initial population contained 10^8 viable spores/ml.

C. botulinum strain 62A spores were prepared in a biphasic culture system as described earlier (8) and were stored at -25 C. They were thawed just prior to use, heat-shocked (80 C for 10 min), diluted in tryptic soy broth supplemented as described below (TSS), and counted as described above. The number of viable spores available for use in each experiment was 10^9 /ml.

Detection media. Tryptic soy broth was pur-

chased with ^{14}C -glucose in a 30-ml volume (culture vial 6A, Johnston Laboratories, Cockeysville, Md.). The vials were inoculated with 6 ml of dilutions of bacterial cultures. They contained 1.42% Trypticase, 0.25% Phytone, 0.42% NaCl, 0.21% K_2HPO_4 , and 0.0139 μCi of ^{14}C -glucose/ml in a final volume of 36 ml. Hereafter, this medium will be referred to as TSB. The ^{14}C -glucose was uniformly labeled with an activity of 3 to 3.5 mCi/0.18 g. For studies with anaerobic organisms, TSB was flushed with sterile N_2 . It would not support detection of low levels of spores of putrefactive anaerobe 3679. Therefore, it was modified as described below to approximate media that enhance germination and outgrowth of anaerobic spores (1, 7, 8).

TSB was supplemented to contain, after addition of bacterial cultures, 3.91% Thiotone, 0.42% yeast extract, and 0.042% sodium thioglycolate in a final volume of 12 or 36 ml. Prior to its addition, the supplement was adjusted to pH 7.2 with 5 N NaOH, and the mixture was autoclaved. Hereafter, this medium will be referred to as TSS (TSB with supplements). It was used with a low or high level of ^{14}C -glucose (0.0139 or 0.0833 $\mu\text{Ci}/\text{ml}$, respectively). Just prior to its use for the detection of anaerobic spores, 0.023 ml of filter-sterilized 5% NaHCO_3 was added per ml of TSS. After addition of a spore inoculum, each vial was rendered anaerobic by sparging the broth with sterile N_2 for 2 min through a sterile cotton-plugged syringe and 3-inch (7.6-cm) 22-gauge needle at a pressure of 10 psi. The excess gas was vented through a second syringe. In early studies, the cultures were mixed with a magnetic stirrer. In later studies, they were mixed on a rotating shaker at 275 rev/min (New Brunswick Scientific Co., New Brunswick, N.J.). There was no apparent difference in detection times.

Beef loaf. Commercial flexible packets of sterile beef loaf were used as the source of food (Oscar Mayer and Co., Chicago, Ill.). They had been stored at 40 to 50 C for 6 years. Each packet contained 391 to 405 g of cooked beef, egg whites, cracker meal made of wheat flour, salt, spice, and monosodium glutamate. A heavy layer of fatty material coated the surface of each loaf. The contents of a packet were placed in a vaccine-capped Waring Blendor and autoclaved at 121 C for 15 min. They were then cooled, stored overnight at 5 C, and mixed with 2 ml of culture which had been grown as described above and diluted in TSS. The cultures were introduced into each Blendor jar by means of a 10-ml syringe with a 13-gauge needle inserted through the vaccine cap on top of the jar. The contents were blended for 1 min and shaken well. Samples (3 ml) were withdrawn from each inverted jar with a sterile syringe and injected into vials which contained 1.0 μCi of ^{14}C -glucose/10 ml of TSS. Triplicate samples were tested separately in this way. The inocula for four different microorganisms ranged from 10^4 to 10^6 viable cells per ml of TSS.

Detection system. Bacterial metabolism was detected in a Bactec model 301 (Johnston Laboratories) by evolution of $^{14}\text{CO}_2$ evolved from the metabolism of ^{14}C -glucose by a culture growing in a vac-

cine-capped bottle. The effluent $^{14}\text{CO}_2$ was trapped in a series of five vaccine-capped Erlenmeyer flasks. The middle three contained 125 ml of 5 M NaOH, and the first and last flasks in the series were empty. The empty flasks minimized possible backflow of fluid into the instrument and loss of fluid droplets containing labeled carbon into the atmosphere. A reading of 20% of full scale was considered as positive evidence of metabolism in the culture vial. This reading is recommended by the manufacturer as sufficiently higher than background to avoid false positives. Background readings above 4% of scale were not observed in this laboratory.

RESULTS

Detection of *S. aureus* and *S. typhimurium*. Cells of *S. aureus* (10^9 to $10^4/\text{ml}$) or *S. typhimurium* (2×10^9 to $1 \times 10^4/\text{ml}$) were inoculated into a final volume of 36 ml of TSB containing 0.5 μCi of ^{14}C -glucose or TSS containing 3.0 μCi of ^{14}C -glucose (0.0139 and 0.0833 $\mu\text{Ci}/\text{ml}$, respectively). Detection times ranged from 4 to 10 hr and from 3 to 9 hr for the high and low levels of inocula of the two respective organisms (Fig. 1). Each 10-fold increment of cells reduced detection time by 1 to 2 hr. Detection time was generally 1 hr shorter in TSS, which contained a higher ^{14}C -glucose level than did TSB. Only with inocula of less than 10^2 salmonellae were detection times about equal in both types of media.

Detection of spores of putrefactive an-

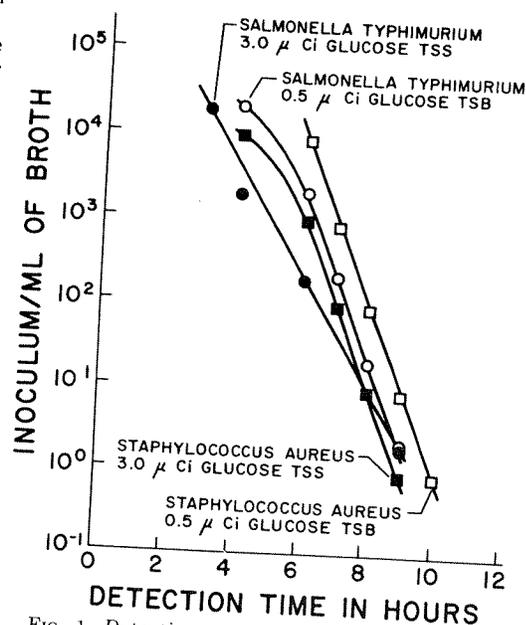


FIG. 1. Detection of *Salmonella* and *Staphylococcus* with 0.5 μCi of ^{14}C -glucose/36 ml of TSB or 3.0 μCi of ^{14}C -glucose/36 ml of TSS.

aerobe 3679 and *C. botulinum*. Heat-shocked spores of putrefactive anaerobe 3679 (10^5 /ml) were inoculated into 36 ml of TSB containing $0.5 \mu\text{Ci}$ of ^{14}C -glucose/ml ($0.0139 \mu\text{Ci}/\text{ml}$). The vials were rendered anaerobic by flushing with N_2 . The activity of $^{14}\text{CO}_2$ evolved over an 8-hr period did not exceed background levels. Similar results were attained over a 28-hr period of incubation when TSB was supplemented with 0.5% yeast extract and 0.7 ml of 5% NaHCO_3 and was inoculated with 3×10^6 to 1×10^4 spores/ml of medium.

Interesting results were obtained when TSS, containing a comparable level of labeled glucose, was used as a detection medium. $^{14}\text{CO}_2$ was produced in amounts that raised activity above background, but below the level set as positive evidence of growth (20% of scale). The readings reached a peak of 0.150 to 0.160 (15 to 16% of scale) at 7 to 8 hr. Results obtained when 3×10^4 spores were inoculated/ml of TSS are shown in Fig. 2. Readings attained a maximum of 0.140 (14% of scale) within 7 hr. Between 7 and 10 hr, the level of activity decreased. Methylene blue smears were made from samples taken from vials at each sampling interval and were examined under phase-contrast microscopy. A number of spores showed loss of refractility, indicating germination. Although no attempt was made to quantitate results, many spores showed outgrowth, but few dividing vegetative cells were observed. Among samples taken thereafter, stainable dividing vegetative cells were rarely observed.

In view of these results, it seemed that a

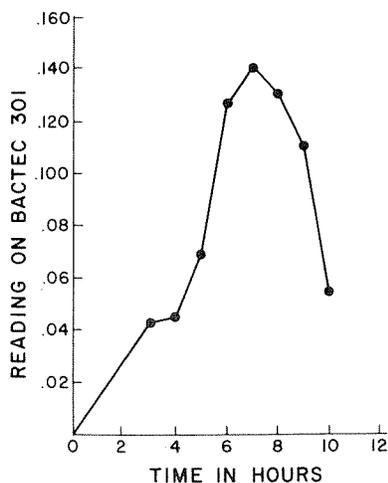


FIG. 2. Activity of putrefactive anaerobe 3679 spores in $0.5 \mu\text{Ci}$ of ^{14}C -glucose/36 ml of TSS.

higher level of ^{14}C -glucose in TSS might allow positive detection of anaerobic spores as defined in this system. Dilutions of heat-shocked spores of putrefactive anaerobe 3679 and *C. botulinum* were made in TSS and inoculated into a final volume of 12 ml of TSS containing $1.0 \mu\text{Ci}$ of ^{14}C -glucose ($0.0833 \mu\text{Ci}/\text{ml}$). This represented a sixfold increase in the level of available labeled glucose compared to previous experiments. Detection times ranged from 4 to 11 hr for inocula of 9×10^5 to 9×10^1 putrefactive anaerobe 3679 spores per ml of broth (Fig. 3). For *C. botulinum*, they ranged from 1 to 8 hr with inocula of 2×10^7 to 2×10^3 spores/ml. The detection times of comparable numbers of spores of the two species were essentially the same.

Cell number at time of detection. The number of cells present at the time of detection in 12 ml of TSS containing $1.0 \mu\text{Ci}$ of ^{14}C -glucose ($0.0833 \mu\text{Ci}/\text{ml}$) showed variations among the species tested (Table 1). Generally, fewer cells of *S. typhimurium* and putrefactive anaerobe 3679 were present at detection (10^5 to 10^6 /ml), whereas there were more cells of *Staphylococcus* (10^6 to 10^7 /ml) and *C. botulinum* (10^7 to 5×10^7). The time required to detect comparable inocula of vegetative cells of either aerobic species was 3 to 4 hr less than

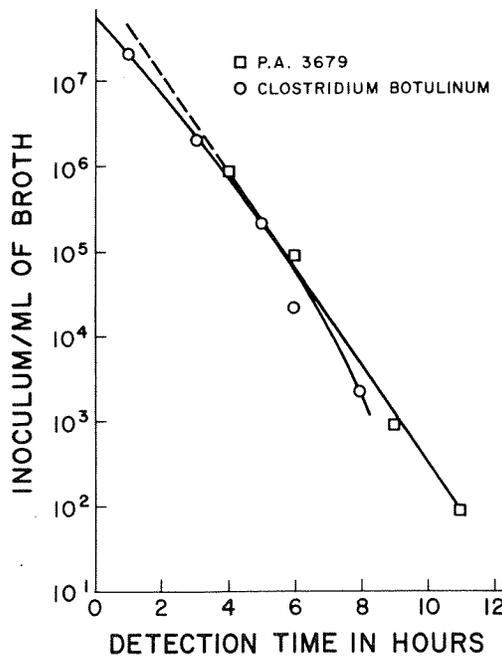


FIG. 3. Detection of putrefactive anaerobe 3679 and *Clostridium botulinum* spores with $1.0 \mu\text{Ci}$ of ^{14}C -glucose/12 ml of TSS.

that required for heat-shocked spores of either anaerobic species (Fig. 1 and 3).

Detection in beef loaf. In these experiments, 10^4 to 10^6 cells of each organism were blended with nonlabeled TSS in sterile beef loaf. Triplicate 3-ml samples were inoculated into separate vials containing 10 ml of TSS and 1.0 μCi of ^{14}C -glucose. Detection times were 2 hr for 5×10^4 salmonellae inoculated per ml of TSS, 4 hr for 3×10^4 staphylococci, 5 hr for 4×10^5 putrefactive anaerobe 3679 spores, and 5 to 6 hr for 10^6 *C. botulinum* spores (Table 2).

DISCUSSION

Variations in detection time of aerobes in TSS and TSB are probably due to the higher level of ^{14}C -glucose in TSS (Fig. 1). Experiments in which the ^{14}C -glucose level in TSB was increased to 0.0833 $\mu\text{Ci/ml}$ (as it was in TSS) also allowed detection times to be decreased. TSB as supplied did not have unlabeled glucose added, but did contain Phytone, which has 37% carbohydrate (BBL; unpublished data). A peptone source with less carbohydrate (such as Thiotone) might afford labeled glucose greater opportunity to be metabolized. A medium different in composition from TSB could shorten detection time. It is clear that 0.0139 μCi of ^{14}C -glucose/ml of TSB or TSS does not seem to provide sufficient label for detection of some species of microorganisms (Fig. 2). Since background activity did not exceed 4% of scale in this laboratory, the maximal readings attained under the conditions illustrated in Fig. 2 (14% of scale) are indicative of active bacterial metabolism. This was confirmed by the observation of germinating spores and dividing vegetative cells under phase microscopy. However, the amount of glucose in the media seemed to be limiting, and the process of germination, emergence, and outgrowth did not lead to active vegetative cell division and detection. A significant amount of the label may have been incorporated into cell constituents and may not have been metabolized to $^{14}\text{CO}_2$. A sixfold increase

in the level of labeled glucose (to 0.0833 $\mu\text{Ci/ml}$ of media) allowed detection of anaerobic spores (Fig. 3). It indicates again that the amount of glucose may be a limiting factor in detecting certain bacterial species.

Others have reported that *C. botulinum* vegetative cells or spores can release $^{14}\text{CO}_2$ without evidence of growth in thioglycolate media (4). Unfortunately, details concerning these results were not provided. In the present experiments, 10^7 heat-shocked *C. botulinum* spores could be detected within 1 hr (Fig. 3). It has been demonstrated that near maximal germination can be obtained within this time under optimal conditions (8). When sufficient labeled glucose is available, it may be used as an energy source during aerobic germination (5). However, the detection time of 10^2 to 10^4 anaerobic spores/ml of TSS was about 3 to 4 hr greater than that of comparable numbers of aerobic vegetative cells (Fig. 1 and 3). This corresponds to the amount of time required for the majority of spores to germinate, emerge, elongate, and initiate cell division (7).

Some investigators failed to detect some bacterial species using a radiometric technique (10). They indicated that their use of a rotary shaker rather than a magnetic stirrer to agitate cultures during incubation probably was not responsible for differences between their results and those of De Land and Wagner (3, 4). In the present experiments, either system of agitation provided equivalent results with the species tested. However, the failure of these investigators to detect growth of *Bacteroides*, as they admit, was probably due to lack of anaerobiosis. Also, they used thioglycolate in their medium, an agent that may inhibit some anaerobes (Difco Manual, 9th ed., p. 195, 1964). Furthermore, their failure to detect inocula of fewer than 10 cells of *Pseudomonas* or group D streptococci in simulated blood samples could be due to sampling errors inherent in handling so few bacteria or to provision of less than optimal conditions for radiometric detection, or to a combination of these factors.

TABLE 1. Number of cells per milliliter at detection with 1.0 μCi of ^{14}C -glucose per 12 ml of TSS

Organism	Range in no. of cells/ml of TSS
<i>Salmonella typhimurium</i>	1.2×10^5 - 3.0×10^6
<i>Staphylococcus aureus</i>	2.3×10^6 - 1.2×10^7
Putrefactive anaerobe 3679	4.5×10^5 - 9.6×10^5
<i>Clostridium botulinum</i>	1.6×10^7 - 5.2×10^7

TABLE 2. Detection of bacteria mixed with beef loaf

Organism	Inoculum per ml of TSS	Detection time (hr)
<i>Salmonella typhimurium</i>	5.3×10^4	2
<i>Staphylococcus aureus</i>	2.9×10^4	4
Putrefactive anaerobe 3679 spores	4.2×10^5	5
<i>Clostridium botulinum</i> spores	1.5×10^6	5-6

The data reported here indicate that 8 to 10 hr was required for detection of inocula of fewer than 10 staphylococci or salmonellae/ml of TSS or TSB (Fig. 1). Furthermore, if individual readings of $^{14}\text{CO}_2$ evolved from one culture over several hours were added, the total activity exceeded that necessary for detection. Therefore, delay in sampling until 6 hr would probably provide positive readings sooner than when earlier and more frequent samplings are used. Cultures often showed turbidity at the time of detection with cell numbers present ranging from 10^5 to 5.2×10^7 /ml (Table 1). However, fresh samples of blood, urine, food, or other natural substances can alter broth chemically or physically, or both, rendering turbidity a poor indicator of bacterial growth.

Washington and Yu (10) used inocula of 1 ml of simulated blood culture for the radiometric analyses and 10 ml for their conventional detection technique. The sampling procedure certainly biased their results in favor of the latter technique. Furthermore, their simulated blood cultures were diluted with Mueller-Hinton broth prior to inoculation. This medium contains a significant amount of carbohydrate. Data with putrefactive anaerobe 3679 show unequivocally that the ratio of labeled to nonlabeled glucose can be critical for detection of some species (Fig. 2 and 3). Media containing nonlabeled carbohydrate can increase time for detection because of competition between labeled and nonlabeled substrates. It may well be that for some bacterial species the optimal amount of label per milliliter is higher than that which has been used in some radiometric studies. One might also consider incorporation of other labeled substrates in addition to ^{14}C -glucose in the event that the test organism does not utilize glucose preferentially as an energy source or that it uses pyruvate anaerobically as a terminal electron acceptor, thereby not generating CO_2 from glucose.

The detection of aerobic vegetative cells of *Salmonella* and *Staphylococcus* and anaerobic spores of putrefactive anaerobe 3679 and *C. botulinum* mixed in broth or blended in beef loaf was achieved readily in TSS containing a

high level of ^{14}C -glucose (Fig. 3 and Table 2). Further exploratory studies should be made to apply these findings to detection of a variety of aerobes and anaerobes from different sources and to optimize the media used for their detection.

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beef loaf

Detection
time
(hr)

2
4
5
5-6