Hazard Inherent in Microbial Tracers: Reduction of Risk by the Use of Bacillus stearothermophilus Spores in Aerobiology

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The use of a “biological tracer” forms an essential part of many aerobiological experiments. Where release of such tracers is likely to result in deliberate or inadvertent human exposure, safety becomes a primary consideration in the selection of the tracer organism. Of the three most commonly used organisms, namely Bacillus subtilis, Escherichia coli, and Serratia marcescens, only the first comes near to satisfying the need for nonpathogenicity and even it has been incriminated as a cause of human infection, sometimes with a fatal outcome. The relevant characteristics of B. stearothermophilus were, therefore, investigated. Because it can grow only at elevated temperatures (minimum 41 C; optimum 56 C), it should not pose a threat to human health and this view is supported by experimental evidence to be presented. It is extremely easy to grow and maintain in the laboratory, and spore suspensions are easily prepared and stored. It withstands the stresses of aerosolization and sampling and its stability in the aerosol state compares favorably with that of B. subtilis var. niger.

Many aerobiological investigations involve the use of a “biological tracer” organism under conditions where human exposure is either inevitable or is likely to occur. In studies involving the development and testing of apparatus for aerosol generation and sampling (2, 6, 19, 20, 26), for room air sterilization (22, 28), or the testing of ventilation systems (5, 14, 21, 34) such circumstances frequently arise and the potential pathogenicity of the tracer organism is a matter of the first importance. If a bacterial tracer is used as an indicator of air movement within the hospital environment (5, 21, 30, 31), the additional hazard of patient exposure is introduced.

The organisms most commonly used as tracers are Bacillus subtilis var. niger (B. globigii), Serratia marcescens, and Escherichia coli. Other organisms have rarely been used, although B. mycoides was selected by Buckley and Tyrrell (7, 8) for direct administration to volunteers to trace the dispersal of nasal secretions and Staphylococcus citreus by Rubbo, Stratford, and Dixson (30, 31) for tracing spread of organisms from textiles in hospital wards.

Of these organisms, E. coli is the most frequent individual cause of infection among hospital patients (33) and the hazard of serious infection due to S. marcescens is now well recognized (1, 13, 29). Neither of these organisms is acceptable for release where human exposure may occur and their use in a hospital setting, where high-risk patients may be exposed, is totally indefensible. Only B. subtilis comes close to fulfilling the requirements of nonpathogenicity, but even it has been incriminated, as have other “nonpathogenic” aerobic spore-bearers, in human infections. These, reviewed by Cox, Sockwell, and Landers (10) and Conrod, Leadley, and Eickhoff (9) have included infections of the eye, generalized infections, urinary tract infections, pneumonia, postoperative wound infections, septicaemia, and infections of the central nervous system. A fatal outcome has been not uncommon. Infections caused by the closely allied organism B. cereus have been reviewed by Farrar (16) and Conrod et al. (9).

In view of these reports, it must be accepted that even B. subtilis possesses some infective potential. While it is true that the risk to
normal healthy people is probably negligible and the organism is normally present in the air, the general public contains many people, both diagnosed and undiagnosed, with a pulmonary disease such as bronchiectasis which could promote infection after exposure to abnormally high doses of B. subtilis. In the authors' opinion, therefore, the release of this organism to expose an unwitting public, especially hospital patients, is unjustifiable. Neither were we willing to accept the potential hazard to laboratory personnel handling experiments in which bacterial clouds must be generated.

A search accordingly was made for a substitute whose growth requirements were such that they could not be met within the tissues of the body and B. steaothermophilus was selected for study. The present report covers those properties of this organism considered relevant to its use as a stable tracer and show it to be almost ideal for this purpose. It must, however, be remembered that repeated inhalation of any antigen may give rise to allergic sensitization, as in "farmer's lung" (27), and in sensitized individuals small doses of inhaled antigen may precipitate an asthmatic response (23). This particular hazard is inherent in the use of any biological tracer.

MATERIALS AND METHODS

Cultures. The strain of B. steaothermophilus studied was isolated and characterized in our laboratory. It was selected for this purpose when, on comparison with three other known strains of this organism, it was found to give better spore yields. This strain was designated "Ottawa 70."

A spore suspension of B. subtilis var. niger was obtained through the courtesy of the Defense Research Board of Canada.

Media. Difco tryptic soy agar and tryptic soy broth were used throughout this study. The formula for the sporulation medium was adapted from Finley and Fields (17).

Determination of colony-forming units. Titrations were performed by making serial tenfold dilutions and plating samples of at least 3 × 1 ml of each dilution on the surface of solid medium in standard petri dishes. Distilled water was used as diluent for spores and normal saline for vegetative suspensions. Colony counts were performed after 18 to 24 hours of incubation at 56 C for B. steaothermophilus and at 37 C for B. subtilis.

Toroid drum studies. A 300-liter rotating drum (18) was used for toroid drum studies. The construction and operation of this apparatus have been described in detail by Mitchell and Brunner (24).

Henderson apparatus. The details of construction and operation of the Henderson apparatus (19) used for the aerosol challenge of mice have been described by Mitchell et al. (25).

Mice. Three-week-old male Swiss albino mice were used (average weight 25 g).

Spore production. The technique for spore production employed in this study was essentially the same as that described by Finley and Fields (17).

Roux bottles, each containing approximately 150 ml of the sporulation medium, were inoculated with 4 ml of an 18 to 24-hour broth culture. After the inoculum had been spread evenly over the agar surface, the bottles were incubated at 56 C. Stained smears from representative cultures were examined periodically to determine the extent of sporulation. Incubation for 5 to 7 days resulted in 80 to 90% sporulation, and at this point the cultures were harvested.

Each culture was flooded with 50 ml of sterile, distilled water. The surface growth was gently loosened with a rubber policeman, and the resulting suspension was centrifuged at 1,500 × g for 10 min. The pooled sediment was washed twice with and finally resuspended in 50 ml of distilled water.

The suspension was treated with the Fisher ultrasonic probe for 30 sec at maximum capacity. This treatment broke up cell clumps without damage to the spores but did not completely eliminate the vegetative cells. To obtain a stable spore suspension free of living vegetative cells, it was necessary to heat it for 30 min at 100 C before storage in a refrigerator.

Air sampling. The slit sampler (model FB-100A; Reyniers & Son, 3806 N. Ashland Ave., Chicago, Ill.) was used to determine the normal thermophile content of the atmosphere. Two-hour samples were taken at the airflow rate of 1 ft³ (28.3 liters) per min, collecting the impacted particles on a petri dish containing tryptic soy agar. The sampler slit, 0.005-inch (0.15 mm) wide, was kept at a slit-to-agar distance of 0.078 inch (2.0 mm).

The Shipe impinger (32) was used to sample aerosols held in the toroid drum. A 10-ml amount of distilled water was used as the collecting fluid and sampling was carried out at an airflow rate of 10 liters per min.

RESULTS

Growth curve. The normal growth curve of strain Ottawa 70 at 56 C in broth is shown in Fig. 1. The inoculum consisted of spores from a suspension in distilled water stored in a refrigerator.

It will be seen that spore germination is very rapid and the lag phase does not exceed 60 min. Replication to a peak concentration of 10⁴ colony-forming units per ml occurs by 12 hr, representing a mean doubling time of about 24 min. The fall in colony-forming unit (CFU) count between 12 and 15 hr is a consistent feature, but its cause has not been investigated.

Thermal death-point of vegetative cells. For tracer work, it is necessary to obtain a stable spore suspension free of vegetative cells. This is most easily achieved by thermal inacti-
vations and the latter. A series of four experiments was carried out to determine the inactivation time of vegetative cells at 100°C in distilled water. The phenomenon of heat activation of spores (see below) made it impossible to measure the inactivation of the 20% of vegetative forms present in the final spore suspension, so these experiments were carried out on 24-hr suspensions of vegetative cells in the resting phase before significant sporulation had occurred.

For practical purposes, heating at 100°C for 30 min leaves a negligible residue of vegetative forms (Table 1), and this time-temperature combination was selected for the preparation of spore suspensions.

**Heat activation of spores.** Curran and Evans (11, 12) reported activation of spores, including those of *B. stearothermophilus*, by heating at sublethal temperatures, and the phenomenon was further investigated by other workers (15, 17). Because heating at 100°C for 30 min had been selected as the inactivation temperature for vegetative cells, the effect on the viable count of heating a spore suspension of the Ottawa 70 strain at this temperature was studied. In a series of four experiments, samples of a spore suspension in distilled water, previously stored in the refrigerator for a period of three months, were heated in a boiling water bath and sampled at intervals for two hours. The pooled results are presented in Fig. 2, where each point on the curve represents the mean count derived from the four individual experiments.

Activation is clearly evident and rises to a maximum level within 10 min. These results agree with those of Curran and Evans (11, 12) and Desrosier and Heiligman (15), but differ from those of Finley and Fields (17) who observed heat-induced dormancy after heating at 100°C, activation occurring only above this temperature with maximal effect at 110°C. The discrepancies are probably due to strain differences and other factors such as the conditions of growth of the bacteria from which the spores were derived.

The time required for the return of the viable spore count to its former level was not investigated. In short-term experiments using a stored suspension, it is unlikely that results would be significantly affected even if the suspension were used before complete stabilization had occurred. Investigators should, however, be aware of the phenomenon lest it should be important in special circumstances.

**Normal thermophile content of the atmosphere.** The effective use of a tracer organism in a bacteriologically uncontrolled environment demands its easy recognition in mixed culture. With *B. subtilis var. niger*, recognition depends on its characteristic colony coloration, and selective media may also be em-

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**Table 1.** Inactivation of the vegetative cells of *Bacillus stearothermophilus* at 100°C in distilled water.

| Inactivation time (min) | Survival (%) |
|------------------------|-------------|
| 5                      | 4.2         |
| 10                     | 2.4         |
| 20                     | 0.6         |
| 30                     | 0.2         |
| 60                     | 0           |
| 90                     | 0           |

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**Fig. 1.** Growth of *Bacillus stearothermophilus* at 56°C in broth.

**Fig. 2.** Heat activation of *Bacillus stearothermophilus* spores at 100°C in distilled water.
ployed. With *S. marcescens*, colony color is also the critical factor. Other organisms such as *E. coli* demand the use of chemically selective or indicator media. In the case of *B. stearothermophilus*, the temperature of incubation, 56 C, provides the selective factor and so the normal thermophile content of the atmosphere was examined. A series of Reyniers samples were taken and the plates incubated at 56 C for 24 hr. The consolidated results are shown in Table 2.

It may be seen that, at two different periods of the year, the thermophile content of the atmosphere was low. During the spring observations, it consisted mainly of easily recognizable actinomycetes. It is clear that the counts are so low, less than 0.2/ft³, that no interference with the interpretation of tracer experiments need be anticipated.

**Minimal temperature for growth.** The rationale for the selection of an obligate thermophile as a nonpathogenic tracer depends upon the supposition that thermal requirements for its replication cannot be satisfied in the human body. It was, therefore, necessary to determine the minimum temperature for growth of the selected strain of *B. stearothermophilus*. The relevant results of a series of experiments to determine this minimum are shown in Fig. 3.

It was found that, in broth culture, replication occurred at 41 C (105.8 F), but not at 40 C (104 F). At 41 C, 3 days were required for detection of significant replication as opposed to fewer than 3 hr at 56 C, and the peak concentration achieved was 1,000-fold lower.

The findings suggest that no normal person nor any patient who does not have a sustained pyrexia of over 105 F could be at risk from this organism, no matter what other predisposing conditions might exist. It need hardly be stated that sustained pyrexia of 106 F is excessively rare.

**Pathogenicity for mice.** The authors are unaware of any report of human infections due to *B. stearothermophilus*, but pathogenicity of an organism for man cannot easily be determined directly.

Experiments were, however, undertaken to determine whether pathogenicity of this organism for mice could be demonstrated either by the intraperitoneal or the respiratory (aerosol) routes of infection.

For challenge by the intraperitoneal route, spore suspensions of three different concentrations (10³, 10⁴, and 10⁷ spores per 0.2 ml) were tested. Each mouse was inoculated with 0.2 ml of the appropriate suspension, and the control mice received an equivalent amount of distilled water.

The exposure of mice to spore aerosol was carried out in the Henderson apparatus (19) by using only the suspension with the highest spore concentration. The mice were exposed to the aerosol for 30 min, control mice receiving a similar exposure to distilled-water aerosol.

To determine the inhaled dose of spores per mouse, the concentration of spores in the Henderson box was assessed by means of two AGI-30 impingers containing distilled water as the collecting fluid. The number of spores inhaled per mouse during the 30 min exposure

![Fig. 3. Minimum incubation temperature for growth of Bacillus stearothermophilus in broth.](image-url)
was thus estimated to be about $5 \times 10^5$.

All mice were kept under observation for 3 weeks, at which point the experiment was terminated because of the absence of any apparent signs of infection. The pooled results of three individual experiments are summarized in Table 3.

Although it was found difficult to recover the deposited spores quantitatively from lung tissue suspensions, it was observed that the number of recoverable spores decreased progressively until none could be detected at 72 and 96 hr after exposure to the aerosol (Fig. 4).

The low recovery at 10 min after exposure (less than 5% of the estimated inhaled dose) may be due to failure to achieve efficient dispersal of organisms retained in the lungs and must be regarded as a minimum figure only. However, the zero counts obtained at 72 and 96 hr strongly suggest that total elimination had occurred by 72 hr. Long-term experiments were not undertaken, but our results suggest that the organism is unlikely to become established in the lung.

It was concluded that even under the max-

**TABLE 3. Effect of intraperitoneal and respiratory challenge of mice with Bacillus stearothermophilus**

| Challenge route                  | Challenge suspension | Mortality or morbidity after 3 weeks |
|----------------------------------|----------------------|------------------------------------|
|                                  | Control (distilled water 0.2 ml) | Spores (per 0.2 ml)  | 10^0 | 10^1 | 10^2 | 10^3 |
| Intraperitoneal                   | 20                   | 30 | 30 | 30 | 0    |
| Respiratory (exposure to aerosol for 30 min) | 15                   | 30 | 60 | 0    |

*Figures represent the consolidated numbers of mice challenged in three separate experiments.
*Estimated inhaled dose: $5 \times 10^4$ spores.

**FIG. 4. Recovery of B. stearothermophilus spores from mouse lung tissue after aerosol challenge.**
with aging of the aerosol.

These experiments suggest that, over the period tested, *B. stearothermophilus* should prove at least as satisfactory as *B. subtilis*.

**DISCUSSION**

The ideal properties of a general purpose bacterial tracer are easy to state. It should be: (i) easy to cultivate for preparative purposes, (ii) rapidly cultivated on isolation by sampling, (iii) easily selected and identified on culture, (iv) stable on storage, (v) stable in aerosol, and (vi) totally nonpathogenic for man and animals.

Of the mesophilic tracers, *E. coli*, *S. marcescens*, and *B. subtilis var. niger*, all satisfy the first three requirements, and *B. subtilis* also satisfies the fourth and fifth. It is our opinion, however, that none truly satisfies the requirement for nonpathogenicity. *B. stearothermophilus* appears to be acceptable in all respects. Being stable, easily handled, and completely safe, *B. stearothermophilus* spores appear to be an ideal "tracer" for general use.

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