CRITERIA FOR THE IDENTIFICATION OF BACILLUS ANTHRACIS

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The identification of Bacillus anthracis involves its differentiation from other aerobic sporeformers, particularly Bacillus cereus. Smith et al. (1952), in a study of aerobic sporeforming bacteria, concluded that B. anthracis is a pathogenic variety of B. cereus. They also stated that strains of B. anthracis which had lost their virulence could not be differentiated from B. cereus. This apparent relationship between B. anthracis and B. cereus was supported by Brown et al. (1958).

Burdon (1956) listed characteristics for the identification of pathogenic strains of B. anthracis, but indicated that attenuated and avirulent strains presented properties closely approaching those of B. cereus.

The report which follows presents data showing that the combination of a few simple tests serves to identify both virulent and avirulent strains of B. anthracis and in addition differentiates them from B. cereus, Bacillus cereus var. mycoides, Bacillus thuringiensis, and Bacillus subtilis. Data will be presented to show that pathogenic strains of B. anthracis which lose their pathogenicity remain B. anthracis and can be differentiated from B. cereus and the other sporeformers tested. Such a differentiation is of taxonomic and medical importance.

MATERIALS AND METHODS

Motility. Motility was determined by inoculating tubes of motility test medium (Difco) to which 0.005 per cent of 2,3,5-triphenyltetrazolium chloride was added to facilitate reading. Tubes were incubated at 37 C for 48 hr. Motility was indicated by the spreading of growth beyond the line produced by the inoculating needle.

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Hemolysis. The hemolytic activity of the organisms was determined by streaking cultures on blood agar base medium (Difco) containing 2 per cent fresh washed sheep cells or 5 per cent fresh beef blood. The use of 5 per cent fresh whole blood (rabbit, sheep, or beef) is recommended. Plates were incubated at 37 C for no longer than 24 hr and examined for the presence of definite zones of hemolysis surrounding the individual colonies.

"String-of-pearls" reaction (Jensen and Klee- meyer, 1953). The "string-of-pearls" reaction was observed by streaking young cultures of the organism to be tested on tryptose agar containing 0.5 unit of penicillin per ml of medium and incubating the plates at 37 C for 3 to 6 hr. A cover slip was placed over the streaked area which was then examined under the oil immersion or high dry objective for the presence of enlarged, round, cellular forms characteristic of the positive reaction. Plates which gave a negative reaction were reincubated for a total of 8 hr before re-examination. Microscopic evidence of growth must be present before a negative result can be reported. Plates incubated overnight may or may not show macroscopic evidence of growth depending upon the presence of penicillin-resistant organisms. Whether resistant or not, enlarged, round forms will be present if the culture is B. anthracis. This procedure has given positive results with two strains of B. anthracis resistant to approximately 100 and 1000 units of penicillin per ml, respectively.

Lysis by gamma bacteriophage (Brown and Cherry, 1955). Eighteen-hour heart infusion agar slant cultures were streaked on tryptose agar plates with sterile cotton swabs. A drop of gamma bacteriophage (10^9 particles or more per ml) and a drop of sterile water to serve as a control were added to separate areas of each streaked plate. Plates were incubated at 37 C for 18 to 24 hr and examined for the presence of lytic zones.

Growth on bicarbonate medium under CO2 (Thorne et al., 1952; Chu, 1952). In most of the
work reported here, nutrient agar containing 10 per cent sterile horse serum and 0.5 per cent sodium bicarbonate was inoculated and incubated under 10 per cent CO₂. In the other tests, nutrient agar containing 0.5 per cent sodium bicarbonate (no serum added) was inoculated and incubated in an atmosphere of 20 per cent CO₂. Both procedures gave similar results. Cultures were streak-plated on either medium, incubated 24 to 48 hr at 37 C, and examined for the presence of rough or mucoid colonies. Colonies of *B. anthracis* which are rough when incubated in air, become mucoid or remain rough (depending upon their virulence) when incubated in the presence of CO₂. Avirulent variants were obtained from the mucoid, virulent *B. anthracis* colonies by incubating the plates for 48 to 72 hr or longer until rough outgrowths appeared at the edges of the mucoid colonies. Repeated platings on these media were carried out where necessary to assure purity of the colonial type.

**Virulence determinations.** All cultures were prepared as spore suspensions by first inoculating casein acid digest broth (Roth *et al.*, 1955) and incubating on a shaker at 37 C for 18 to 24 hr. The cultures were then incubated overnight in a 48 C water bath in order to break up chains. They were then heat shocked at 65 C for 30 min. The resulting spore suspension was plated to determine the viable spore count. Appropriate dilutions were then prepared for inoculation into mice.

White mice weighing 18 to 24 g each were assigned in a random manner to cages until groups of 10 mice were obtained. A minimum of 4 groups of 10 mice each were injected intraperitoneally with 0.5 ml of saline suspensions containing graded doses of each bacterial strain. Ten control mice were each injected with 0.5 ml of sterile saline. The LD₅₀ values were calculated from the number of animals which died within 7 days. The virulence of all cultures except *B. subtilis* was determined.

**Cultures.** Sixty-six cultures of *B. anthracis*, 3 of *B. thuringiensis*, 16 of *B. cereus* var. *mycoides*, 10 of *B. subtilis*, and 36 strains labeled *B. cereus* were received from the American Type Culture Collection, the N. R. Smith Collection (from Dr. W. C. Haynes), and from Fort Detrick sources. In addition, 44 avirulent variants were obtained from 44 virulent strains of *B. anthracis*. The total number of strains tested and reported was 175.

**RESULTS**

**Identifying characteristics.** The characteristics chosen for the identification of *B. anthracis* and for its differentiation from *B. cereus* and other nonanthrax sporeformers were "string-of-pearls" formation and susceptibility to gamma bacteriophage. Supporting, but not absolute characteristics were lack of motility and lack of hemolysis. Morphology on bicarbonate agar and virulence determinations are characteristics which vary according to the virulence of the strain of *B. anthracis*. The results obtained with the various bacilli are listed in table 1. The trend is quite apparent. All strains received as *B. anthracis* were nonmotile, nonhemolytic, gave a positive "string-of-pearls" test, and were lysed by the specific

**TABLE 1**

Reactions of Bacillus strains (labeled as received)

<table>
<thead>
<tr>
<th>Strains</th>
<th>No. of Strains</th>
<th>Motility</th>
<th>Hemolysis</th>
<th>&quot;String-of-Pearls&quot;</th>
<th>Gamma Phage Susceptibility</th>
<th>Bicarbonate Agar + CO₂</th>
<th>Virulence (LD₅₀ less than 10⁶ spores)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. anthracis</em></td>
<td>66</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>53</td>
</tr>
<tr>
<td><em>B. cereus</em>†</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>60</td>
<td>6</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>32</td>
<td>0 4</td>
<td>0 4 4</td>
<td>4</td>
<td>0 0</td>
<td>4 4 4 0</td>
<td>0 32</td>
</tr>
<tr>
<td><em>B. cereus</em> var. <em>mycoides</em></td>
<td>16</td>
<td>0 1 16</td>
<td>0 0 0</td>
<td>0</td>
<td>0 0</td>
<td>0 0 32</td>
<td>0 16</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>3</td>
<td>0 3 0</td>
<td>0 0 3</td>
<td>0</td>
<td>0 0</td>
<td>0 3 0</td>
<td>0 3</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>10</td>
<td>0 4 0</td>
<td>0 0 10</td>
<td>0</td>
<td>0 0</td>
<td>0 0 10</td>
<td>0</td>
</tr>
</tbody>
</table>

* M = mucoid colonies; R = rough colonies.
† Two strains reclassified as *Bacillus anthracis*, and two were found to be contaminated with *Bacillus anthracis* (see table 3).
‡ = Not done.
bacteriophage. Depending upon their virulence, they produced rough or mucoid colonies on bicarbonate agar and gave a mouse virulence reaction as indicated. Only strains labeled and finally classified as B. anthracis showed LD50 values of less than 10^4 spores. The LD50 values obtained with the various organisms are presented in Table 2. The majority of the mucoid B. anthracis strains gave LD50 values of 10^6 to 10^8 spores, whereas the majority of rough B. anthracis strains as well as a majority of the other strains tested showed LD50 values of 10^4 or more spores. It should be noted that some overlapping of LD50 values does occur between the various strains in Table 2. Calculation of 95 per cent confidence limits of the strain of B. anthracis with an LD50 of 10^4 spores showed an overlap with the other strains having LD50 values of 10^6 spores.

The nonanthrax species tested varied with respect to motility and hemolysis but were generally positive in these two characteristics. None of the strains labeled B. cereus (except 4 cultures described below), B. cereus var. mycoides, B. thuringiensis, and B. subtilis gave a positive "string-of-pearls" reaction, was lysed by gamma bacteriophage, or produced mucoid colonies on bicarbonate agar. When an intraperitoneal dose of less than 10^4 spores was selected as an arbitrary dividing line between virulent and avirulent cultures, all of the above nonanthrax strains had LD50 values of 10^6 spores or more.

**Reactions of mislabeled cultures.** Thirty-six cultures labeled B. cereus were received and tested. Four of these (ATCC 6630 and 6472, and NRS 722 and 723) each yielded strains which did not conform to the characteristics of B. cereus but which did conform to the characteristics of virulent B. anthracis (isolate I in Table 3). Two of these original cultures, ATCC 6630 and ATCC 6472, also contained B. cereus (isolate II). In order to determine whether or not the two strains present in ATCC 6630 were related, the mucoid virulent B. anthracis strain was incubated on bicarbonate agar plates until a rough outgrowth occurred. This rough outgrowth was isolated, purified, and tested. Although it was greatly reduced in virulence, the rough isolate was unchanged in its characteristics of negative motility, negative hemolysis, positive "string-of-pearls" reaction, and positive phage susceptibility. In other

<table>
<thead>
<tr>
<th>Strain</th>
<th>10^6</th>
<th>10^7</th>
<th>10^8</th>
<th>10^9</th>
<th>10^10</th>
<th>10^11</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus anthracis mucoid*</td>
<td>8</td>
<td>34</td>
<td>11</td>
<td>6</td>
<td>1</td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>B. anthracis rough* (rough when received)</td>
<td>5</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>B. anthracis rough (derived from mucoid strains)</td>
<td>3†</td>
<td>1†</td>
<td>1</td>
<td>2</td>
<td>18</td>
<td>5</td>
<td>44</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>4</td>
<td>17</td>
<td>5</td>
<td>6</td>
<td>1</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>B. cereus var. mycoides</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>

* On bicarbonate medium.
† Received as Bacillus cereus; reclassified as Bacillus anthracis (see Table 3).

**Table 2**

**Mouse intraperitoneal LD50 values**

**Table 3**

**Reactions of four cultures labeled Bacillus cereus**

<table>
<thead>
<tr>
<th>Code on Label</th>
<th>Isolates</th>
<th>Motility</th>
<th>Hemolysis</th>
<th>String-of-Pearls</th>
<th>Phage Susceptibility</th>
<th>Morphology on Bicarbonate Agar under CO2</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRS 722*</td>
<td>I</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Mucoid</td>
</tr>
<tr>
<td>NRS 723†</td>
<td>I</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Mucoid</td>
</tr>
<tr>
<td>ATCC 6630§</td>
<td>I</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Rough</td>
</tr>
<tr>
<td>ATCC 6472¶</td>
<td>I</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Mucoid</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Rough</td>
</tr>
</tbody>
</table>

* NRS 722 is ATCC 944.
† Isolates I were identified as Bacillus anthracis.
‡ NRS 723 is ATCC 4509.
§ ATCC 6630 is NRS 305.
¶ ATCC 6472 is NRS 701.
words, the rough *B. cereus* strain in all probability did not arise from any change in the *B. anthracis* strain, but was a contaminant.

"When a strain of *B. anthracis* loses its pathogenicity is it still the anthrax bacillus or is it *B. cereus*?" (Smith et al., 1952). In an attempt to answer this question, 44 mucoid strains of *B. anthracis*, showing mouse intraperitoneal LD$_{50}$ values of $10^4$ to $10^5$ spores, were plated on bicarbonate agar, and incubated until rough outgrowths appeared at the edges of the mucoid colonies. Rough variants were isolated, purified, and then tested. One rough colony was isolated from each virulent strain, giving 44 virulent (LD$_{50}$ values of $10^4$ to $10^5$)-avirulent (LD$_{50}$ values of $10^4$ to $10^5$) pairs. Only those pairs are reported in which the 95 per cent confidence limits showed statistically valid decreases in the virulence of the rough mutants. Although the rough variants showed a decrease in virulence, they showed no change in motility, hemolysis, "string-of-pearls" reaction, or susceptibility to gamma bacteriophage.

**DISCUSSION**

The results reported in this study show that *B. anthracis* can be identified and differentiated from *B. cereus* and other sporeforming organisms by the characteristics of a positive "string-of-pearls" reaction and positive gamma phage susceptibility. Negative hemolytic and motility reactions support the identification, but are not conclusive diagnostic characters. After identification has been made, virulence determinations and morphological appearance on bicarbonate agar serve to characterize the organism further. In testing 175 cultures of the genus *Bacillus*, the reactions of these cultures fell into two groups: namely, *B. anthracis* and non-*B. anthracis*. No cultures originally labeled or finally identified as *B. anthracis* were found to be either motile or hemolytic, whereas other sporeformers were generally positive in these reactions. Only cultures identified as *B. anthracis* gave positive "string-of-pearls" and positive gamma phage susceptibility reactions. A rapid indication of the identity of a suspected colony can be achieved in 3 to 6 hr time with the "string-of-pearls" reaction. Although no single reaction should be relied upon for identification of this organism, the reactions reported in this paper will readily differentiate *B. anthracis* from *B. cereus*.

The isolation of an aerobic sporeformer which produces mucoid colonies on bicarbonate agar incubated under CO$_2$ is strongly indicative of *B. anthracis*. Although *B. anthracis* produces either mucoid or rough colonies under these conditions, depending upon its virulence, none of the other cultures tested produces mucoid colonies. Burdon (1956) reported that *B. cereus* did not form mucoid colonies on bicarbonate agar.

The results of our study disagree with those reported by Brown et al. (1955) with respect to motility, hemolytic activity, and interpretation of virulence data. We found no motile strains of *B. anthracis*. However, Brown et al. (1955) referred to the production of motile strains of *B. anthracis* induced by exposure to omega bacteriophage. Six of these motile strains were tested by Sterne and Proom (1957) and found to consist of 4 strains of *B. subtilis* and 2 strains of *B. cereus*. The possible occurrence of motile contaminants in a study of this kind must be borne in mind. Until such time as motile strains are definitely identified as *B. anthracis*, we agree with Sterne and Proom (1957) that the existence of motile strains of *B. anthracis* has not been conclusively demonstrated. Brown et al. (1958) reported that 45 of 120 strains of *B. anthracis* were hemolytic, whereas our studies have shown that *B. anthracis* is not hemolytic. The age of the erythrocytes, the presence of added substances such as glucose in the blood, and incubation periods extended beyond 24 hr can result in the occurrence of zones of hemolysis with *B. anthracis*. It is our opinion that hemolysis tests made in the manner described by us will give negative results with *B. anthracis*.

Virulence determinations are of importance in the study of *B. anthracis*, but are of no value in identifying avirulent strains for such strains show LD$_{50}$ values as high as $10^9$ spores. The words "virulent" or "avirulent" have little meaning unless stated in quantitative terms. Virulence determinations which neglect to quantitate the inoculum, number of animals inoculated, and the resulting number of deaths are difficult to evaluate. The mouse is an acceptable animal to use for the determination of virulence with *B. anthracis* as reported here. Results obtained in this study were clear-cut and meaningful, when quantitative procedures were employed. The determination of mouse intraperitoneal LD$_{50}$ values will indicate the presence of *B. anthracis* if virulence is arbitrarily accepted at an LD$_{50}$ value of less than $10^4$.
spores. Reliance should not be placed on this single determination even though all cultures in our study which showed a virulence of this order were B. anthracis.

Apparently, a major reason for the classification of B. anthracis as a variety of B. cereus (Smith et al., 1952; Brown et al., 1958) is the concept that B. anthracis differs from B. cereus only in pathogenicity. When pathogenicity is lost, these authors considered it difficult or impossible to differentiate between the two strains. Our study has shown that when virulent B. anthracis cultures became avirulent, each avirulent culture retained its original B. anthracis characteristics with the exception of morphology on bicarbonate agar, which has been associated with virulence. Indeed, it would be surprising if B. anthracis were so unusual that it alone did not have an avirulent counterpart so common in pathogenic microorganisms. The results reported here show that B. anthracis, regardless of pathogenicity, can be differentiated from B. cereus and is a separate and distinct species. Thus B. anthracis should not be considered a variety of B. cereus. It is recognized that it might be possible by genetic techniques to induce in B. anthracis, such B. cereus-like characteristics as motility, hemolysis, and resistance to gamma phage. Work of this nature should be performed with strains containing genetic markers to rule out the possibility of contamination with other aerobic sporeformers.

ACKNOWLEDGMENTS

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SUMMARY

Evidence was presented to show that Bacillus anthracis could be identified and differentiated from Bacillus cereus and other sporeformers by a positive "string-of-pearls" reaction and a positive reaction with gamma bacteriophage. The absence of motility and hemolysis, and a characteristic morphology on blood or nutrient agar media served to support the identification of B. anthracis.

No motile or hemolytic strains of B. anthracis were found. The non-B. anthracis sporeformers tested were generally positive in these two characteristics. All strains of B. anthracis gave positive "string-of-pearls" and bacteriophage susceptibility reactions, whereas all of the other strains gave negative reactions. Virulent strains of B. anthracis produced mucoid colonies on bicarbonate agar incubated in CO<sub>2</sub>; the other strains never showed mucoid colonies in CO<sub>2</sub>, when the colonies were rough in air.

The necessity for quantitative virulence determinations was indicated and its value in the identification of B. anthracis discussed. Forty-four avirulent mutants were derived from 44 virulent strains of B. anthracis and in each instance no change occurred in reactions for motility, hemolysis, "string-of-pearls," and bacteriophage susceptibility.

The evidence reported here indicates quite strongly that Bacillus anthracis is not a variety of Bacillus cereus and that virulent or avirulent B. anthracis can be differentiated from B. cereus. B. anthracis should not be classified as a variety of B. cereus.

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