GERMINATION OF SPORES OF CERTAIN AEROBIC BACILLI UNDER ANAEROBIC CONDITIONS

NORMAN G. ROTH AND DAVID H. LIVELY
Camp Detrick, Frederick, Maryland

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Previous work by this laboratory (Roth et al., 1955) has established that a definite aeration requirement existed for spore formation of Bacillus anthracis and Bacillus globigii. This aeration requirement was shown to be different at various stages of growth. In the case of spore germination of the aerobic organisms tested, the present work indicates that the necessity for aerobic conditions is completely lacking.

Many early reports have suggested that aerobic spore-forming bacteria could grow under anaerobic conditions in canned foods. Weinzierl (1919) was among the first to show that aerobic spore formers were associated with spoilage in canned foods; but he believed that this type of spoilage was generally associated with air leaks. Savage (1923) was unable to prevent the growth of Bacillus subtilis and Bacillus mesentericus under the most rigid anaerobic conditions he could devise; as a result, he concluded that spoilage in canned foods could be caused by aerobic spore formers in the absence of air. Cheyney (1919) reported that B. mesentericus did not appear to be strictly aerobic. In a series of experiments with B. subtilis and B. mesentericus, Bushnell (1922) found these organisms capable of growth to some extent in what he considered to be as complete anaerobic conditions as it was possible to obtain.

Basset (1930) claimed that he observed growth of B. anthracis anaerobically and that its cultures sporulated after incubation in the absence of air in 6 days.

The present study has shown that spores of certain aerobic organisms will germinate readily under anaerobic conditions. However, in our work, no appreciable growth or sporulation in the absence of air over extended incubation periods has been demonstrated.

MATERIALS AND METHODS

Organisms used. The test organisms were a typical virulent strain of Bacillus anthracis, a yellow-orange pigmented strain of Bacillus subtilis var. niger (formerly identified as Bacillus globigii) and the ATCC 10778 strain of Bacillus megaterium. The strain formerly identified as B. globigii has been shown to blacken tyrolyne agar and, in general, have the other characteristics of B. subtilis var. niger described by Smith et al. (1952).

Inoculum and incubation conditions. In all cases the inoculum consisted of a spore suspension which was 5 per cent by volume of the medium; this suspension had been autolyzed at 47 C for 18 hours and heat-shocked 30 minutes at 65 C. The heat-shocked spore suspension was washed three times in distilled water. Cultures were incubated at 34 C in 250-ml Erlenmeyer flasks.

Methods of maintaining anaerobiosis. In one series of experiments anaerobiosis was maintained with 0.5 per cent sodium thioglycolate; in the second series anaerobiosis was maintained by incubation under an atmosphere of nitrogen.

The medium employed for the first series of experiments was the casein acid digest medium described by Roth et al. (1955), modified as indicated, with 0.5 per cent sodium thioglycolate to obtain anaerobic conditions. As an indicator of oxidation-reduction potential, 0.01 per cent resazurin was used. Eh measurements were made on a Beckman pH meter using a platinum-calomel electrode system. Special care was taken to avoid agitation of the medium during Eh readings.

In the second series of experiments anaerobic conditions were maintained by incubation under nitrogen. Flasks containing casein digest medium were flushed rapidly with nitrogen for 10 minutes; during this process agitation on a reciprocating shaker at 99 3-inch strokes per minute was maintained. The nitrogen flow then was reduced to a level where constant bubbles were seen and maintained at this level throughout the test. In order to remove any traces of oxygen which might have been mixed with the nitrogen, a modification of the procedure recommended by Neish (1952) was employed. In our modification, the gas was
first passed through a pyrex tube packed with hot reduced copper wire, heated with a bunsen burner. The nitrogen was cooled by passage through a condenser prior to being introduced into the test system.

After incubation, anaerobic cultures, both in the sodium thioglycolate and nitrogen tests, were stoppered with rubber stoppers and sealed with paraffin. The aerobic control cultures in all cases were shaken at a rate that resulted in an oxygen level approximately 1.0 mM O₂/L/ min as determined by the sulfitic method (Bartholomew et al., 1950).

Method of determining germination. The germination of spores was determined by increase in heat sensitivity after incubation and by microscopic examination of Schaeffer-Fulton spore stains.

Immediately after incubation the cultures were heat-shocked at 65°C for 30 minutes. Spore germination was determined as a decrease in spore count since the vegetative cells were killed by the heat shock treatment. To determine per cent germination, the following formula was used:

\[ \text{percent germination} = \left( \frac{\text{viable spore count after incubation}}{\text{viable spore count at 0 time}} \right) \times 100 \]

Viable spore counts were determined by standard plate count techniques. All plate counts reported were averages of counts obtained on triplicate plates.

Germination was determined microscopically with smears stained by the Schaeffer-Fulton technique. In the zero time cultures, no red-stained vegetative cells were observed. Any appearance of red-stained cells after incubation was taken as an indication of germination. No attempt was made to determine per cent germination by this method.

RESULTS

Aerobic Germination. An attempt was made to establish the aeration levels necessary for germination of spores of the genus *Bacillus* in a series of tests with various oxygen levels as determined by the sulfitic method. Within 1 hour, at all levels of air from 0.1 to 1.5 mM O₂/L/ min., it was found that *B. subtilis* var. *nigre* and *B. anthracis* showed greater than 99 per cent and greater than 90 per cent germination, respectively. In additional experiments, essentially complete spore germination also occurred within 1 hour in stationary flasks that were sealed with paraffin immediately after seeding; only the amount of air trapped in the flasks was available for the process of germination.

The results led to an investigation of germination by the bacilli under anaerobic conditions.

Anaerobic Germination. *A. Sodium thioglycolate medium.* A series of spore cultures of the three test organisms were inoculated into anaerobic media; anaerobiosis was maintained with 0.5 per cent sodium thioglycolate. The germination obtained in 1 hour under this condition was compared with that obtained with control spore cultures inoculated into aerobic shake flasks receiving approximately 1.0 mM O₂/L/ min as determined by sulfitic tests and into non-nutrient anaerobic controls. To determine the per cent germination, cultures were allowed to incubate for 1 hour at 34°C. At the end of the incubation period, they were heat-shocked for 30 minutes at 65°C and viable spore counts were made.

The results of three replications with each of the test organisms are shown in Table 1. It can be seen that the germination obtained under anaerobic conditions was of the same order of magnitude as that under aerobic conditions; greater than 90 per cent germination was observed within 1 hour with all organisms under both conditions. By microscopic examination large numbers of red-stained vegetative cells were observed after the 1-hour incubation in casein digest medium both with and without sodium thioglycolate. The stock spore suspension contained 100 per cent green-stained spores.

In non-nutrient control cultures either no germination or very little germination was observed. The germination reported in some of these cases may have been due to inadequate washing of the stock spore suspensions. In some cases, a few red-stained cells were observed in the field of predominantly green-stained spores, indicating that some germination had occurred in these control cultures.

It was found that when the incubation period was increased to 2 or 4 hours, no increase in germination in either the aerobic or anaerobic cultures was noted. Evidently germination proceeded to the limits of the experimental conditions within the period of the 1-hour test.

*B. Nitrogen atmosphere.* An additional test was performed to ascertain if the germination observed actually was occurring under anaerobic
TABLE 1
Germination of spores of certain aerobic bacilli under aerobic or anaerobic incubation as indicated
Anaerobic conditions were maintained with 0.5 per cent sodium thioglycolate

<table>
<thead>
<tr>
<th>Organism</th>
<th>Medium</th>
<th>Incubation Condition</th>
<th>Viable Spores per ml × 10⁸ After Indicated Incubation at 34°C</th>
<th>Per Cent Germination After 1-Hr Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis var. niger</td>
<td>H₂O + 0.5% CH₂SHCOONa</td>
<td>Anaerobic non-nutrient</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>Casein digest</td>
<td>Aerobic, nutrient</td>
<td>1000</td>
<td>11000</td>
</tr>
<tr>
<td></td>
<td>Casein digest + 0.5% CH₂SHCOONa</td>
<td>Anaerobic, nutrient</td>
<td>1000</td>
<td>11000</td>
</tr>
<tr>
<td>Bacillus anthracis</td>
<td>H₂O + 0.5% CH₂SHCOONa</td>
<td>Anaerobic, non-nutrient</td>
<td>1000</td>
<td>10000</td>
</tr>
<tr>
<td></td>
<td>Casein digest</td>
<td>Aerobic, nutrient</td>
<td>1000</td>
<td>30100</td>
</tr>
<tr>
<td></td>
<td>Casein digest + 0.5% CH₂SHCOONa</td>
<td>Anaerobic, nutrient</td>
<td>1000</td>
<td>60100</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>H₂O + 0.5% CH₂SHCOONa</td>
<td>Anaerobic, non-nutrient</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Casein digest</td>
<td>Aerobic, nutrient</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>Casein digest + 0.5% CH₂SHCOONa</td>
<td>Anaerobic, nutrient</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* In general all plate counts were made at a dilution which resulted in 30 to 300 colonies per plate. Counts were all converted to the same log base for ease of reporting.

TABLE 2
Germination of spores of certain aerobic bacilli under aerobic or anaerobic incubation as indicated
Anaerobic conditions were maintained under a nitrogen atmosphere

<table>
<thead>
<tr>
<th>Organism</th>
<th>Medium</th>
<th>Incubation Atmosphere</th>
<th>Viable Spores per ml × 10⁸ After Indicated Incubation at 34°C</th>
<th>Per Cent Germination After 1-Hr Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 hr</td>
<td>1 hr</td>
</tr>
<tr>
<td>Bacillus subtilis var. niger</td>
<td>H₂O</td>
<td>Air</td>
<td>2000</td>
<td>2000</td>
</tr>
<tr>
<td></td>
<td>Casein</td>
<td>Air</td>
<td>2000</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>digest</td>
<td>N₂</td>
<td>2000</td>
<td>1</td>
</tr>
<tr>
<td>Bacillus anthracis</td>
<td>H₂O</td>
<td>Air</td>
<td>700</td>
<td>700</td>
</tr>
<tr>
<td></td>
<td>Casein</td>
<td>Air</td>
<td>700</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>digest</td>
<td>N₂</td>
<td>700</td>
<td>50</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>H₂O</td>
<td>Air</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Casein</td>
<td>Air</td>
<td>90</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>digest</td>
<td>N₂</td>
<td>90</td>
<td>4</td>
</tr>
</tbody>
</table>

* In general all plate counts were made at a dilution which resulted in 30 to 300 colonies per plate. Counts were all converted to the same base log for ease of reporting.

conditions; a series of tests were performed in the same manner using stationary cultures incubated under an atmosphere of nitrogen. Control cultures consisted of suspensions incubated in water and in shake flasks at an aeration level equivalent to 1.0 ms sulfite oxygen/L/min. The results of one typical run with each test organism are shown in table 2. It can be seen
that anaerobic germination, of the same order of magnitude as that in table 1, was observed under the nitrogen atmosphere.

Anaerobic Sporulation. An attempt was made to obtain anaerobic sporulation of the aerobic bacilli as reported by Basset (1933). Under the conditions of our experiment, no sporulation of anaerobic cultures of the test organisms was obtained within 8 days at 34°C. Cultures receiving the equivalent of 1.0 mm sulfite oxygen/L/min in the same medium all showed excellent growth and essentially complete sporulation within 24 hours.

DISCUSSION

The present studies have established that under the experimental conditions described, certain bacilli were capable of germinating rapidly but were not capable of sporulating after extended incubation under aerobic conditions. Anaerobiosis was maintained by use of sodium thioglycollate as a reducing substance in the medium or by incubation under an atmosphere of nitrogen.

Brewer (1940) demonstrated that 0.1 per cent sodium thioglycollate was sufficient to maintain anaerobic conditions in liquid media for long periods without a seal or other special apparatus. Eh measurements on our CAD media containing 0.5 per cent sodium thioglycollate indicated that the Eh was approximately −0.3V at the time of inoculation. Although no Eh measurements were made during the germination period, resazurin, which was used as an O/R indicator, remained in the colorless dihydroresorufin state throughout the tests, indicating that anaerobic conditions were maintained.

Wynne et al. (1952) reported germination under aerobic conditions of spores of Clostridium perfringes, Clostridium chauvei, and putrefactive anaerobe No. 3679. Earlier work by Knight and Fildes (1930) established the upper limit for germination of spores of Clostridium tetani, at pH 7.0 to 7.65 in an otherwise favorable medium, at Eh ±0.11V.

With the aerobic organism, Bacillus mycoides, Knaysi (1945) observed germination of spores in the presence of a fermentable sugar under anaerobic conditions. We have found in our studies that three typical aerobic spore-forming organisms germinated readily at negative Eh values. Apparently, the aeration requirements for germination of the Bacilli and Clostridia are not critical. Spores of the aerobic organisms tested are capable of germinating at the anaerobic Eh values. The work of Wynne et al. (1952) and Knight and Fildes (1930) indicates that spores of the anaerobic organisms, conversely, may be able to germinate at aerobic Eh values.

An unresolved question as to the role of Eh vs. oxygen in growth of aerobic spore formers still exists in the literature. Knaysi and Dutky (1934) reported that in their experiments the limiting factor in the growth of B. megaterium in vacuum was oxygen and not the Eh of the medium. On the other hand, Wood et al. (1935) indicated that the oxidation-reduction conditions of the medium determined the growth of B. megaterium in the presence of adequate oxygen.

Because of this disagreement, it was considered desirable to repeat our germination experiments by incubation in an atmosphere free of oxygen. Anaerobic conditions were maintained by bubbling a constant stream of oxygen-free nitrogen through the medium. A high percentage of germination, of the same order as with sodium thioglycollate, was observed under these conditions.

ACKNOWLEDGMENT

Grateful acknowledgment is made to Dr. Howard M. Hodge, Camp Detrick, for his sustained interest and criticism.

SUMMARY

Greater than 90 per cent germination of spores of Bacillus anthracis, Bacillus subtilis var. niger, and Bacillus megaterium was observed to occur within 1 hour in a casein digest medium at negative Eh values. Greater than 90 per cent germination of spores of these organisms was also observed to occur within 1 hour in a casein digest medium held under an atmosphere of oxygen-free nitrogen. No increase in germination was noted upon incubation for periods longer than 1 hour. No sporulation of these organisms was observed to occur under anaerobic conditions after incubation for 24 hours or 6 to 8 days.

REFERENCES


