Physiological Studies of an Oligosporogenous Strain of *Bacillus popilliae*

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A relatively small but consistent increase in the frequency of spore formation by an oligosporogenous strain of *Bacillus popilliae* (NRRL B-2309M) was obtained by adding 0.1% sodium pyruvate to the sporulation medium. The frequency of spore formation was essentially the same when a low level of glucose, trehalose, or glucose-6-phosphate or a high level of α-methyl-D-mannoside was added as the carbon and energy source. Many other variations in the cultural medium and cultural conditions failed to enhance spore formation of 2309M, and no spores were found in four asporogenic strains under any of the conditions tried. There were no significant differences between the 2309M strain and three nonsporulating cultures with respect to (i) the rate and extent of growth, (ii) the rates of glucose utilization, or (iii) volatile acid production and utilization. None of the cultures tested was found to produce detectable levels of extracellular protease or an antibiotic. The only consistent marker found associated with spore formation was the development of catalase activity, and this activity was stimulated by heating at 80°C for 10 min. This was not found unless morphological evidence of spore formation was observed. The germination of the spores formed by 2309M in vitro was stimulated by heat shock and by the addition of pyruvate to the germination medium.

The isolation of a variant culture of *Bacillus popilliae* (NRRL B-2309M) which sporulated at significant frequencies in vitro (22) provided the opportunity to compare some of its physiological properties during spore formation with the parent and other strains which did not produce spores under the same conditions. It was hoped that such studies would provide information which could be used in devising conditions for sporulating the wild-type cultures in vitro. McKay et al. (10) demonstrated that oligosporogenous variants all are able to oxidize acetate, whereas cultures initiated from spores of the wild-type strain do not. However, other asporogenous variants oxidize acetate even more rapidly than the strains producing spores. Therefore, although the derepression of acetate oxidation may be an important factor, it obviously does not constitute the only difference between strains sporulating in vitro and those which do not.

This report covers the results of many experiments conducted to compare the physiological properties of the sporogenic strain of Sharpe et al. (22) with asporogenous strains of *B. popilliae*. Studies involved the influence of various energy sources on sporulation; comparisons of growth rates, glucose utilization, pH changes, and volatile acid production; and the production of sporulation-specific enzymes and antibiotics during colonial development on the sporulation medium. Also, some observations of the germination of spores produced in vitro were made.

**MATERIALS AND METHODS**

**Cultures and cultural methods.** All parent cultures of *B. popilliae* used were obtained from Northern Utilization Research and Development Division, Agricultural Research Service, Peoria, Ill. Also, spores of NRRL B-2309 produced by injection of Japanese beetle larvae were supplied by Grant St. Julian, Jr., of the above laboratory. The oligosporogenous strain used was NRRL B-2309M (22), and the asporogenous strains were NRRL B-2309S, B-2309PA, B-2309N, and B-2309MC. These strains will be referred to in this paper as 2309M, 2309S, etc. The history of the strains used and procedures used for their maintenance have been described (10).

**Growth and sporulation.** The basic sporulation medium was that described by Sharpe et al. (22) except that the 0.05% trehalose was replaced by 0.05% glucose. The medium contained 1% Mueller Hinton broth medium solids (Difco), 1% yeast extract (Difco)
co), 0.3% K$_2$HPO$_4$ and 0.05% glucose and will be referred to as MYPG. Pyruvate (0.1%) was added to this medium where indicated. The substitution of glucose for trehalose was made after we found that it did not influence the sporulation frequency which we achieved. All of the components of the medium except the agar were filter-sterilized.

Spores used for inoculating plates were harvested from plates by washing with three 5-ml volumes of water and were routinely heat-shocked at 60 C for 10 min. In a few instances (see below), the vegetative cells in spore-containing colonies were destroyed by inverting the petri dish over a piece of chloroform-soaked cotton for 5 min prior to harvesting, and no heat shock was used. The plates were spot-inoculated to obtain 10 evenly spaced colonies per plate. An inoculator was fashioned from a No. 13 rubber stopper with ordinary straight pins stuck into it. After sterilization, the pin heads were dipped into a suspension of sporules and then touched to the agar surface. Inocula for the asporogenic strains were produced in the Trypticase-yeast extract-glucose (TYG) medium described previously (12).

The frequency of sporulation ([number of spores/total number of cells and sporules] × 100) was determined by direct counting of the number of characteristic spores with parasporal bodies present in a population of at least 200 cells. The suspensions counted were washed from single plates as described above. For estimates of growth by optical density at 620 nm, the suspensions were washed twice with 0.01 m potassium phosphate (pH 7.4) and resuspended in a total volume of 25 ml of the same buffer. A Gilford model 2000 spectrophotometer was used for optical density measurements.

Analytical measurements. Plates containing the MYPG plus pyruvate medium to be used for analysis of changes occurring during colonial development and sporulation were weighed immediately after they were poured and cooled. They were dried for 2 to 3 days and inoculated as described above. One plate was used at each interval during incubation starting at zero time. The cells were wiped from the surface with non-absorbent (delicatesen) paper, and the plate was weighed to determine the loss due to evaporation. The agar was cut into strips, and macerated by forcing it through a 25-ml syringe without a needle. The syringe was rinsed with water equivalent to two volumes of the original weight of the medium and combined in a 250-ml flask with the macerated medium plus enough water to correct for the weight loss during incubation. The stoppered flasks were incubated for 2 hr at 30 C on a rotary shaker to allow for equilibration and allowed to sediment; 25 ml of the aqueous supernatant solution was clarified by centrifugation at 12,000 × g for 10 min.

The pH of this extract was read directly. Pyruvate was assayed by the procedure of Friedemann and Haugen (4), and the colorimetric assay outlined in Neish (14) was used to detect lactate. Glucose utilization was estimated by the reduction in total reducing sugars present. In one experiment, [U-14C]glucose (7,900 counts per min per µmole) was added to the medium, and the decrease in the nonvolatile compounds present was estimated by determining the radioactivity remaining after acidification to below pH 6.0 to liberate dissolved CO$_2$ and subtracting the values obtained for volatile acid. The radioactivity in the aqueous samples was counted in the scintillation solution of Bray (1). A 1-ml sample of the acidified mixture was placed in the outer well of a Conway dish along with 1 ml of concentrated H$_2$SO$_4$. Hydroxide of hyamine (1 ml) was placed in the center well. After 48 hr of incubation, the hydroxide of hyamine was quantitatively removed with methanol and made to a total volume of 2 ml. Portions of this solution were placed in a toluene-based scintillation fluid (11), and the radioactivity was counted. These counts were considered to represent the volatile acid. A Nuclear-Chicago Mark I scintillation spectrometer was used for all 14C determinations.

Catalase production. Cells and spores were harvested from the sporulation medium at various intervals as described above. They were assayed for catalase activity by using the manometric procedure of Lawrence and Halvorson (8) except that the oxygen release during the first 4 min after adding substrate was used to calculate activity. Cell extracts were prepared by exposure to ultrasonic oscillation for 20 min in a 100-w ultrasonic disintegrator (Measuring and Scientific Equipment, Ltd., London). Proteins were estimated on 1 N NaOH extracts (40 C for 2 hr) of hot (90 C for 30 min) 5% trichloroacetic acid extracts of cells by the method of Lowry et al. (9). Protein in cell extracts was estimated by this method without previous extraction. Dry weights of cells and spores were determined after drying at 110 C for 24 hr.

The refractile bodies used were produced as described previously (12). The spores from Japanese beetle larvae were washed in 0.01 m phosphate buffer (pH 7.4) six times before testing for catalase.

RESULTS
Effect of carbon and energy sources on sporulation. During the course of these investigations, a number of different carbon and energy sources were tested for their influence on the frequency of sporulation of strain 2309M (Table 1). It should be noted here that the data given represent the microscopic estimate of the percentage of the population represented by those sporangia containing a characteristic spore and parasporal body. We routinely observed in the spore-containing cultures an equal or higher percentage of other forms which were typical of the refractile bodies described previously (12). The addition of 0.1% sodium pyruvate to the glucose-containing medium had the most pronounced effect on the frequency of spor formation of those tested. However, it was not effective when used alone. A low level of glucose-6-phosphate supported sporulation as well as glucose, but higher levels progressively reduced the frequency of sporulation. In contrast, with α-methyl-D-mannoside, the frequency of sporulation increased as the concentra-
| Compound(s) added\(^a\) | Per cent spores\(^b\) |
|-------------------------|----------------------|
|                         | Control\(^c\) | Treated\(^d\) |
| None                    | 2–6        | <1         |
| Trehalose (0.1%)        | 2–6        | 2–6        |
| Trehalose (0.05%)       | 2–6        | 2–6        |
| Glucose (0.05)          |            |            |
| + Pyruvate (0.05%)      | 3          | 11         |
| + Pyruvate (0.1%)       | 3          | 12         |
| + Pyruvate (0.2%)       | 3          | 12         |
| + Lactate (0.05%)       | 3          | 3          |
| + Gluconate (0.05%)     | 3          | 2          |
| Pyruvate (0.05%)        | 3          | 1          |
| Glucose-6-phosphate     |            |            |
| (0.1%)                  | 5          | 7          |
| (0.25%)                 | 3          |            |
| (0.5%)                  | 1          |            |
| \(\alpha\)-Methyl-D-mannoside |        |            |
| (0.05%)                 | 5          | 1          |
| (0.2%)                  | 2          |            |
| (0.5%)                  | 7          |            |
| (1.0%)                  | 2          |            |

\(^a\) Compounds indicated were added to the Mueller Hinton-yeast extract-phosphate medium.

\(^b\) Per cent sporulation was estimated microscopically after a minimum of 21 days of incubation.

\(^c\) Control medium (MYPG) contained 0.05% glucose.

\(^d\) Medium contained the carbon sources indicated.

Sporulation was increased up to 0.5% but decreased at the 1% level. Growth on the \(\alpha\)-methyl-D-mannoside medium also increased as the concentration of sugar increased. The dry weight of cells in the 10 colonies from one plate of the media containing 0.05, 0.2, and 0.5% of this sugar averaged 2.4, 5.5, and 8.3 mg (dry weight), respectively. This was after 20 days of incubation.

Other attempts were made to increase the frequency of sporulation in this medium but without significant success. These included (i) addition of other compounds as carbon and energy sources including fructose, sucrose, \(\alpha\)-methyl-D-glucose, salicin, soluble starch, and cellobiose; (ii) preparation of MYPG plus pyruvate medium by using the supernatant solution of centrifuged stationary phase broth culture (spent medium) and adding various carbon and energy sources; (iii) feeding of glucose by adding a drop of sterile 0.2% glucose to a sterile filter paper disc on the agar surface at daily intervals; (iv) adding \(10^{-3}\) M adenosine 3',5'-monophosphate (cyclic AMP) initially and also by adding a drop of \(10^{-4}\) M to a filter paper disc as in (ii) at various intervals during colonial development; (v) fractionation of yeast extract into anionic and cationic components by the use of Dowex 50, Cl\(^-\)-form and H\(^+\)-form columns, respectively, and using the concentrates to replace the yeast extract in the medium and as supplements to it; (vi) the addition of reduced glutathione (0.01%) initially and, also, by feeding as in (iii) and (iv); (vii) the addition of 5% sterile defibrinated blood to the agar before autoclaving or by adding various levels of hemin before filtration of the nutrient components; (viii) addition of 0.04% dipicolinic acid; and (ix) addition of a variety of metal ions to the regular medium and to the medium prepared with a solution of Mueller Hinton solids and yeast extract which had been treated with Dowex 50, Na\(^+\)-form resin at pH 6.0 to remove divalent cations. A number of similar experiments were performed with this medium and the yeast extract-peptone medium of Pfeil and Ordal (16) in broth cultures incubated on a shaker without success. Strains of \(B.\) popillae which do not produce spores under the conditions outlined by Sharpe et al. (22) were also tested for sporulation in all of the above experiments without success. When 0.2 to 0.5% salicin was used as the energy source in a broth medium (16), many of the cells of most strains of \(B.\) popillae tested appeared to be swollen in a manner characteristic of sporangia. However, no other evidence of sporulation was noted.

**Growth and glucose utilization during colonial development.** Figure 1 compares the growth rates and rates of glucose utilization of the oligosporogenous strain (2309M) with the ranges observed with three nonsporulating strains. There was a delay of 12 to 18 hr in the initiation of rapid growth of 2309M as compared to the other three. This was expected since a spore inoculum was used with 2309M and a cell inoculum with the other strains. The mean generation time during exponential growth for all strains was estimated at about 3.75 hr, which is considerably longer than that observed in broth cultures (2.5 to 3.0 hr). As in other aerobic bacilli, spore formation occurred in 2309M as the culture approached stationary phase of growth. An occasional swollen cell was observed after 4.3 days of incubation, and the maximum percentage of spores (6%) was observed after 5.1 days of incubation. The period between exponential growth and the stage of maximum growth was prolonged (~3 days). This prolonged period of a declining growth rate was correlated with a constantly decreasing rate of glucose utilization. There was essentially no further increase in cell mass after all of the glucose was exhausted from the medium. The utilization of the added pyruvate paralleled that of glucose.

**Changes in pH and the production of volatile acid**
during colonial development. There was not a sufficient amount of volatile acid accumulated during growth to estimate by steam distillation. Therefore, [U-\textsuperscript{14}C]glucose was added to the MYPG plus pyruvate medium, and the volatile acid was estimated by radioactivity measurements. It is realized that there may be a significant loss of volatile acid during incubation of the plates, but the data should reflect a general pattern. All four strains listed in Fig. 1 were tested, but the results with only two of these will be presented since they are representative. There was nothing unique about the pH and volatile acid patterns of the oligosporogenous strain (Fig. 2). The pH values declined to a minimum of 7.2 to 7.3 during 4 days of incubation and then increased dramatically. Maximum volatile acid levels were observed at 4 days also, but the acid levels declined only slightly thereafter. The total volatile acid accumulated in the asporogenic (2309S) strain was about two times higher than that in 2309M but still represented only about 46% of the nonvolatile \textsuperscript{14}C which had disappeared from the medium. The nonvolatile \textsuperscript{14}C disappeared at a slower rate from 2309M than from 2309S, and the maximum volatile acid observed amounted to only 36% of the nonvolatile \textsuperscript{14}C which had disappeared. Tests for the possible intermediate accumulation of lactate in these cultures were negative. The results with strain 2309N were essentially identical to 2309M, and those for 2309PA were very similar to 2309S.

Spore formation was observed with 2309M (see inset in Fig. 2) after the pH of the medium had started to increase.

Development of sporulation-specific antibiotics and enzymes. All attempts to demonstrate the presence of an antibiotic during sporulation of strain 2309M failed. Staphylococcus aureus, Escherichia coli and Bacillus megaterium were all used as test organisms. Dilute suspensions of the test organism (\(\sim 10^9/\text{ml}\)) were streaked adjacent to colonies of \textit{B. popilliae} at various stages during colonial development and spore formation. In no instance was any evidence of inhibition noted. Similarly, no intracellular or extracellular protease activity was detectable with 2309M at various stages of colonial development. Tests were conducted by (i) adding 1% casein to the sporulation medium and observing for zones of hydrolysis around the colonies, (ii) incubating colonies and the surrounding excised medium or cell extracts with azo-albumin and measuring the release of trichloroacetic acid-soluble chromophoric groups (2), and (iii) measuring the release of 5% trichloroacetic acid-soluble \textsuperscript{14}C from \textsuperscript{14}C-protein during incubation with
TABLE 2. Development of catalase activity during sporulation of Bacillus popilliae (2309M)

| Time of incubation (d) | Microscopic observation | \(O_2\) from \(H_2O_2\) [aliters per min per mg (dry weight)] |
|------------------------|-------------------------|----------------------------------------------------------|
|                        |                         | Unheated | Heated<sup>b</sup> |
| 3                      | All cells               | 0        | 0              |
| 4                      | Few swollen cells       | 0        | 0              |
| 5                      | Few swollen cells       | 0        | 0              |
| 6                      | Few spores (<1%)        | 1.7      | 0.8            |
| 7                      | 2-4% Spores            | 1.1      | 1.0            |
| 10                     | 2-4% Spores            | 1.5      | 1.5            |
| 17                     | 2-4% Spores            | 1.9      | 1.9            |
| 26                     | Cells separated from spores by centrifugation<sup>e</sup> | 2.2      | 2.2            |
|                        | Spores (~80%) after separation from cells<sup>c</sup> | 3.6      | 4.7            |

<sup>a</sup> Cells were harvested from plates at the time indicated and washed one time; catalase was determined manometrically. Reaction mixture contained 0.5 ml of 0.1 m phosphate buffer (pH 7.0), approximately 100 mm \(H_2O_2\), 5 to 10 mg (dry weight) of cells, and water to 3 ml.

<sup>b</sup> 80 C for 10 min.

<sup>c</sup> Nonsporulated cells were separated by centrifuging the suspension for 5 min at 3,000 X g. The cells in the supernatant fluid were then centrifuged at 10,000 X g for 15 min and resuspended. There were no refractile spores evident in this suspension. The spores were concentrated by resuspending the pellet of the 3,000 X g centrifuging in 10 ml of water and repeating the process five times. Each time, the top layer of the pellet which was largely cells was discarded. The percentage of spores in the final suspension was estimated by microscopic examination.

Cells from colonies or with cell extracts. The latter protein was prepared from cells of *B. popilliae* grown in 250 ml of TYG broth to which 25 \(\mu\)Ci of a \(U-^{14}C\) amino acid mixture was added. The radioactive protein was prepared as described by Sadoff et al. (20), and the final solution contained about 1 mg of protein per ml with a specific activity of 1.5 \(\times 10^5\) counts per min per mg. Incubation of 50 \(\mu\)liters of this \(^{14}C\)-protein with 10 \(\mu\)liters of protease from *B. cereus* (20) in a total volume of 0.1 ml for 30 min at room temperature resulted in the release of sufficient trichloroacetic acid-soluble material to give a count of 186 counts/min in a sample equal to 10 \(\mu\)liters of the original reaction mixture. This is equal to about 19% of the total radioactivity. When water was substituted for the protease, an equivalent trichloroacetic acid-soluble sample contained only 14 counts/min of \(^{14}C\).

Incubation of 0.2 ml of the same *B. cereus* protease with 1 ml of azo-albumin solution for 30 min resulted in an increase in the optical density at 340 nm of 0.13.

The only enzyme activity which was consistently associated with sporulation was catalase. Previous data have demonstrated that vegetative cells are devoid of this enzyme (15, 25) but that spores produced in vivo and refractile bodies produced in the laboratory have significant catalase activities (12). Catalase was also produced by strain 2309M during sporulation in colonies (Table 2). It is obvious that catalase activity appeared during sporulation, and that it was completely resistant to heating at 80 C for 10 min. However, the data indicate that it was produced early in the sporulation process. Thus, the cells separated from the spores by centrifugation were about 50% as active as the fraction greatly enriched in spores. No evidence of significant catalase activity was detected with any of the asporogenous strains during colonial growth on the same medium. Two of these strains (2309MC and 2309N) were derived from 2309M.

Comparisons of the catalase activity in the cells and spores of strain 2309M produced in vitro with that of refractile bodies produced as described previously (12) and with spores from Japanese beetle larvae are shown in Fig. 3. Although the total activity of the refractile bodies was high, it was mostly heat-labile. In contrast, the activity associated with the cells and spores harvested from colonies on plates was stimulated by heating at 80 C for 10 min. The partial heat lability of the enzyme observed with the spores from larvae may have resulted from contamination with the heat-labile catalase from larval hemolymph or catalase-producing bacteria. Although the spores were washed six times before testing, there is no assurance that they were free of hemolymph. Also, it is not possible to prevent some degree of microbial contamination of the spores when harvesting from larvae. The catalase associated with the cells, spores, and refractile bodies was destroyed by autoclaving. However, in some experiments, a low background of up to 10% of the total activity was noted after autoclaving. Also, some sensitivity (up to 50%) of catalase to heating at 80 C for 10 min has been observed with cells and spores of 2309M produced in vitro after storage for several weeks at -20 C.

As evident in Table 3, the catalase activity in the spores freed of cells, sporangia, and parasporal bodies appeared higher than observed with an enriched intact spore suspension noted earlier in Table 2. Unfortunately, the spore yields were so low we did not have a sufficient quantity to perform other experiments with them. The cell ex-
tract was fractionated, and the highest catalase activities were found in the supernatant solution after centrifugation at 20,000 × g (Table 3). The activity was not decreased by prolonged dialysis or by precipitation with (NH₄)₂SO₄. The rate of O₂ released from H₂O₂ increased in a linear manner with protein concentration with the dialyzed extract. Also, the enzyme was inactivated by peroxide by a first-order reaction. The activities in all cell fractions were strongly inhibited by azide and cyanide except for that in the 20,000 × g pellet. Thus, it is likely that the activity is primarily due to a heme-type catalase.

**Germination of in vitro spores.** During the isolation of oligosporogenous mutants of *B. popilliae*, it was observed that colonies developed from heat-shocked (60°C for 15 min) samples on plates of MYPG plus pyruvate but not on the same medium without pyruvate (Bhumiratana and Costilow, unpublished data). Plating of heat-shocked and chloroform-treated suspensions of in vitro spores produced on media with and without pyruvate confirmed these observations (Table 4). The numbers of colony-forming units observed with the pyruvate-containing media were from 2 to over 100 times greater than the controls.

Pyruvate influenced both the rate and extent of germination. This was indicated by an experiment in which an equal number of heat-shocked spores (~4 × 10⁴ spores per ml) were added to MYPG

**Table 3. Catalase activity of spores and of various fractions of cells of Bacillus popilliae 2309M**

| Fraction                      | O₂ (μliters per min per mg of protein) |
|-------------------------------|--------------------------------------|
|                               | Control | 10 mM Na₂CO₃ | 10 mM KCN |
| Cells and spores              | 6.6-8.1 | 3.0          | 4.2       |
| Spores                        | 30.0    |              |           |
| Cell extract                  | 4.1     |              |           |
| 2,000 × g supernatant fluid   | 4.1-5.5 | 1.4          | 1.6       |
| 20,000 × g supernatant fluid  | 4.4-5.5 | 0.6          | 1.1       |
| 20,000 × g pellet             | 2.7-3.1 | 1.9          | 2.0       |
| Dialyzed 20,000 × g supernatant fluidΔ (NH₄)₂SO₄ precipitate² | 6.0      | 0.7          | 1.2       |
|                               | 7.6      |              |           |

* Cells and spores were exposed to ultrasonic oscillation for 2 min which was sufficient to break cells and to remove sporangia and parasporal bodies but did not break the spores. The pellet of the 2,000 × g centrifugation contained the spores. The spores were washed three times with water to remove debris before testing.

³ Dialyzed 64 hr against water at 4°C.

⁴ Protein was precipitated from the 20,000 × g supernatant solution with (NH₄)₂SO₄ (90% saturation) and centrifuged; the pellet was dissolved in the original volume of water.
Table 4. Effect of pyruvate on the germination of Bacillus popilliae (2309M) spores

| Mediuma | Expta |
|---------|-------|
|         | I (thousands/ml) | II (millions/ml) | III (millions/ml) |
| MYPG    | 0.5 | 1.2 (7.7%)c | |
| MYPG + pyruvate (0.1%) | 67.5 | 9.4 (60.0%) | 3.6 (18%) |
| TYG     | 4.8 | 1.6 (10.3%) | |
| TYG + pyruvate (0.1%) | |  | 5.7 (29%) |
| Brain heart infusion (BHI) | |  | |
| BHI + pyruvate (0.1%) | |  | 2.4 (12%) |

- a Spores were heated at 60 C for 10 min in experiment I; they were treated with CHCl₃ in experiment II, and were heated at 60 C for 20 min in experiment III.
- b MYPG, 1% Mueller Hinton broth medium solids (Difco), 1% yeast extract (Difco), 0.3% K₂HPO₄, and 0.05% glucose. TYG, Trypticase-yeast extract-glucose.
- c Percentages are of the number of spores in the original suspension determined by a direct microscopic count.

and MYPG plus pyruvate broths and incubated on a shaker, and heat-shocked subsamples were plated at different intervals on the same medium containing pyruvate. The germination rates were low in both media. Thus, the time required for a 90% reduction in the number of heat-resistant spores in the control broth was about 5 hr as compared to 4 hr in the broth plus pyruvate. After 3 days of incubation of these media, about 1.1% (350 per ml) of the original number of colony-forming units were still heat-resistant in the control as compared to 0.2% (75 per ml) in the pyruvate medium.

The in vitro spores of 2309M are activated by heat. A suspension of cells and spores was harvested from a plate of MYPG after 23 days of incubation and plated on JB medium (18) before and after heating at 60 C for 15 min. There were fewer than 10⁴ colony-forming units per ml in the unheated samples compared to 8.3 × 10⁵ in the heated. However, the spores are not very heat-stable. After heating at 60 C for 20 min, the plate count on MYPG plus pyruvate agar of a spore suspension was 8.6 × 10⁴ as compared to 3.1 × 10⁸ and 1.4 × 10⁸ after heating at 80 C for 10 and 20 min, respectively.

**DISCUSSION**

We have never been able to achieve the frequency of spore formation by B. popilliae strain 2309M in vitro as reported by Sharpe et al. (22). They obtained about 20% spores in this medium, whereas we routinely observed 2 to 6%. However, they pointed out that the lot of yeast extract used influenced the results dramatically. Also, they routinely prepared inocula by drying, whereas we either heat-shocked or exposed inocula to chloroform to eliminate vegetative cells. This could also influence the degree of sporulation.

The generation time of B. popilliae cells growing in colonies on an agar medium is somewhat longer than that in broth. However, the greatest difference in development is in the period of time during which the growth rate is deaccelerating. This period is very prolonged when the cells are growing in colonies (3 to 4 days), as compared to a few hours in broth cultures (11). This difference is probably due to the slow diffusion of the energy source to the cells on the agar. Glucose disappeared from the agar at essentially the same time that growth ceased. It is apparent that spore formation occurs with the 2309M strain during the period of a declining growth rate, and we found no increase in the percentage of cells with spores after this point. There was, however, a secondary growth response after some apparent cell lysis. These observations correlate well with those of Sharpe et al. (22). They observed that the spores in the colonies were found in a concentric ring near the outer edge of the colony surrounded by vegetative cells and prespore forms in the outer fringe.

It is known that some species of the Bacillaceae sporulate in the presence of high levels of certain energy sources (5, 11). Hsu and Ordal (5, 6) demonstrated that Clostridium thermosaccharolyticum sporulated well when carbohydrates were present which limited the growth rate or when glucose was fed to the culture at growth-limiting rates. Apparently, this organism requires carbohydrate for energy during sporulation. Sharpe et al. (22) observed that the presence of trehalose at levels above 0.1% greatly reduced the frequency of sporulation with B. popilliae. However, high populations of spores accumulate in the hemolymph of the Japanese beetle larvae in the presence of high levels of trehalose (17). It is possible, of course, that the trehalose present is mostly not available to the cells. Data in this paper indicate that spore formation occurs in a glucose medium when the glucose levels become very low. However, the percentage of cells forming spores in the presence of α-methyl-D-mannoside was at least equivalent to that in a glucose medium. A signifi-
ciant growth response was observed with increasing levels of this sugar. The oxidation of α-methyl-D-
mannoside is inducible in this organism, and it is utilized at much slower rates than glucose (Bhu-
miratana and Costilow, unpublished data). On the
basis of these facts, we propose that B. popilliae
requires the presence of a carbohydrate at concentra-
tions which severely limit growth or in a growth-limiting form for sporulation to occur.

The pH changes in the medium during growth
and sporulation of 2309M are quite similar to
those observed during sporulation of other bacilli
(13, 26). However, the pH is not depressed as
much and rises to higher levels than observed with
other bacilli. In addition to oxidizing accumulated
acetic acid, basic compounds such as ammonia
must either be produced or released from the cells.
Previous data demonstrated that this strain along
with other oligosporogenous strains as well as
some nonsporulating cultures would oxidize acetate and glutamate (10). However, we have
never observed strain 2309S to oxidize these sub-
strates, yet the changes in pH were similar in this
medium.

We were unable to demonstrate the appearance
of extracellular protease or the production of an
antibiotic by the oligosporogenous strain of B.
popilliae. These are early events commonly associ-
ed with sporulation (13, 26). However, our data
are subject to question because of the relatively low
frequency of spore formation obtained. As
pointed out by Schaeffer (21), oligosporogenous
cultures of bacilli may have phenotypes similar
to cultures which sporulate at high frequencies or
to those which form no spores. However, a recent
report indicates that Bacillus brevis sporulates
normally without protease production or signifi-
cant protein turnover (23).

The only unique marker found which was asso-
ciated with in vitro spore formation was the pro-
duction of a heat-stable catalase. Vegetative cells
of B. popilliae have no catalase activity (15, 25)
and they are sensitive to H₂O₂ (3), but spores from
larvae and refractile bodies do have this activity
(12). Catalase was consistently found at the onset
of sporulation of 2309M. It appeared to be pro-
duced at an early stage in spore formation since
it was present in cells separated from spores. This
is one of the first enzymes demonstrated in spores
of aerobic bacilli (8). The catalase found in spores
of B. cereus was stable to 80°C for 10 min and 100
°C for 5 min, whereas vegetative cell enzyme was
completely inactivated by these treatments. Sadoff
(19) observed that the heat stability of catalase in
B. cereus developed at essentially the same time
as glucose dehydrogenase. This is one of the early
sporulation-specific enzymes found in B. cereus.

There is a great amount of evidence that the cata-
lase activity in B. popilliae is sporulation specific;
namely, (i) no activity is observed with 2309M
until morphology changes characteristic of spore
formation are observable, (ii) strains which
do not produce spores, including two variants
derived from 2309M, do not produce catalase
grown under the same conditions as 2309M,
and (iii) all of the catalase activity in sporulating
cultures of 2309M is stable to 80°C for 10 min.

The catalase activity of B. popilliae was inhib-
ited partially by heme poisons, cyanide and azide,
and, thus, appeared to be primarily due to a heme-
iron enzyme. However, its production was not
stimulated by the addition of preformed iron-
porphyrin compounds, hemin or boiled blood, as
in the case of lactic acid bacteria (7). The activity
was not reduced by exhaustive dialysis or by pre-
cipitation with ammonium sulfate.

The germination of B. popilliae spores is quite
slow; 4 to 5 hours is required to reduce the num-
ber of heat-resistant colony-forming units by
90% in a rich medium. Activation of the spores
by the procedure of Splitsstoesser and Farkas (24)
used for spores produced in vivo had no signifi-
cant effect on the extent of germination. The addi-
tion of pyruvate to the germinating medium en-
hanced both the rate and extent of germination.
The presence of this compound in the plating me-
dium enhanced recoveries by about 2- to over
100-fold in different trials. However, the percent-
age of spores present which germinated varied
greatly among experiments and may have resulted
from differences in the age of the suspensions. It
has been demonstrated that spores produced in
vivo germinate more readily after extended stor-
age (24).

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