JOURNAL OF BACTERIOLOGY, Sept. 1967, p. 759-765
Vol. 94, No. 3
Copyright © 1967 American Society for Microbiology
Printed in U.S.A.

Comparisons of Cells, Refractile Bodies, and Spores of *Bacillus popilliae*[1]

BRIJ M. MITRUKA,[2] RALPH N. COSTILOW, S. H. BLACK, AND R. E. PEPPER

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48823, and Department of Microbiology, Baylor University, College of Medicine, Houston, Texas 77025

Received for publication 6 June 1967

Spores of *Bacillus popilliae* from infected larvae and refractile bodies produced in a Trypticase-barbiturate medium were similar but distinct from vegetative cells of this organism in protein, nucleic acid, and enzyme composition. The spores and refractile bodies were found to have catalase activity, some of which was heat-resistant. This enzyme was not found in the vegetative cells. The spores contained dipicolinic acid, but the refractile bodies did not. The latter were similar to cells in having considerably higher levels of phosphate extractable with cold trichloroacetic acid and of poly-β-hydroxybutyrate than had the spores. Electron microscopy demonstrated conclusively that the refractile bodies are distinctly different from either cells or spores of *B. popilliae*. The possibility that these bodies are formed as a result of an aborted sporulation process is discussed.

Practically every cell of *Bacillus popilliae* produces a spore during production of "milky disease" in Japanese beetle larvae. In contrast, the best efforts published to date have resulted in a maximum of about 0.3% sporulation in vitro (30) on a solid medium. More recently, a strain has been isolated (NRRL B-2309M) in which about 15% sporulation has been observed on a solid medium (E. S. Sharpe and G. St. Julian, Bacteriol. Proc., 1967, p. 10).

For several years we have sought to ascertain any unique characters which might provide a key to sporulation of *B. popilliae*. This organism is a strict aerobe (24); glucose is catabolized by both the glycolytic and hexosemonophosphate pathways, producing acetate, lactate, and CO_2 primarily (4); acetate is not oxidized to a significant extent except by a single mutant strain (4); the electron transport system is deficient in cytochrome *c* (5); stationary-phase cultures generate considerable hydrogen peroxide and lack the ability to degrade it (5); cells are quite sensitive to 0.01 M H_2O_2 (8); the organism is auxotrophic for 11 amino acids and for thiamine (36); and barbituric acid is required for consistent growth in a synthetic medium (36).

During these studies, a medium was developed in which phase-refractile bodies were produced by a high percentage of the cells (8). Since these bodies were similar to spores in some characters, it was thought that they might result from an abortive sporulation process. The purpose of this study was to compare these bodies with vegetative cells and spores of *B. popilliae* with respect to composition and structure.

MATERIALS AND METHODS

Culture, cultural methods, and preparation of extracts. B. popilliae B2309-P-A and spores of *B. popilliae* harvested from Japanese beetle larvae were obtained from the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill. The culture was maintained in broth as described previously (4). Cells were produced in a 1.5% Trypticase (BBL), 0.5% yeast extract, 0.2% glucose, and 0.6% K_2HPO_4 medium (TYG) at *p*H 7.4, as described previously (8).

Refractile bodies were produced in the "sporulation medium" as described by Costilow et al. (8); this medium is now designated TB medium. Unless otherwise specified, the inoculated medium was supplemented with 0.02% DL-sodium-β-hydroxybutyrate after 3 days of incubation, since this was found to stimulate the formation of refractile bodies (Mitruka and Costilow, *unpublished data*). The refractile bodies were separated from the residual cells by centrifuging at 3,000 × *g* for 15 min as described previously (8).

The spores harvested from hemolymph from larvae were washed six times and the vegetative cells and refractile bodies were washed twice with distilled water. Cell extracts were prepared with the Nossal cell

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article 4058.

[2] Present address: Laboratory of Soil Microbiology, Department of Agronomy, Cornell University, Ithaca, N.Y. 14850.

disintegrator (McDonald Engineering Co., Cleveland, Ohio) as described previously (25).

Analytical methods. Proteins were estimated on 1 N NaOH extracts (40 C for 2 hr) of hot (90 C, 30 min) trichloroacetic acid-insoluble material by the method of Lowry et al. (20). This method was also used to estimate the protein in extracts prepared by mechanical disruption. Results obtained by digestion of the trichloroacetic acid-insoluble fraction by micro-Kjeldahl and subsequent nesslerization by the method of Johnson (15) were similar (±10%). Ribonucleic acid (RNA) was estimated on hot trichloroacetic acid extracts by the orcinol method (22), and deoxyribonucleic acid (DNA), by the diphenylamine reaction (6), with calf thymus DNA as the standard.

Poly-β-hydroxybutyrate (PHB) was estimated by the procedure of Law and Slepecky (18). Dipicolinic acid (DPA) was measured by 270 mμ absorbancy of ether extracts prepared by the procedure of Perry and Foster (26). Colorimetric assays (14) of the ether extracts yielded similar results. Total acid-soluble phosphate was determined according to the procedure of Fiske and SubbaRow (12), on the cold (3 C, 15 min) 5% trichloroacetic acid-soluble fraction of disrupted vegetative cells, spores, and refractile bodies prepared as described above.

The following enzymes were assayed by the procedure indicated through use of crude extracts (centrifuged at 3,000 × *g* for 15 min): adenosine deaminase (27), alanine racemase (34), glucose dehydrogenase (2), pyrophosphatase (20), aldolase (32), enolase (5), lactic dehydrogenase (17), condensing enzyme (23), aconitase (1), diaphorase (25), cytochrome *c* reductase, and succinic cytochrome *c* reductase (11). A suspension of the particles sedimented at 3,000 × *g* for 15 min was used for determination of ribosidase by the procedure of Powell and Hunter (7). β-Hydroxybutyrate (BHB) dehydrogenase was determined on the soluble fraction of extracts centrifuged 90 min at 120,000 × *g* by the procedure of Delafield et al. (9). Both soluble and particulate fractions were assayed for reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase by the spectrophotometric assay (10). Catalase of intact cells, refractile bodies, and spores was measured manometrically in a Warburg apparatus. Reaction mixtures contained 100 mM H_2O_2, 10 mM phosphate buffer (*p*H 6.8), and the respective cells. The amount of oxygen released within the first 4 min was used to calculate the activity because of the rapid inactivation which occurred in the presence of H_2O_2.

Microscopy. Washed vegetative cells of *B. popilliae* B-2309-P-A, refractile bodies produced in TB medium, and spores harvested from hemolymph of infected larvae were photographed on Plux-X Kodak film through phase-contrast optics of a Zeiss microscope. Portions of these same cell preparations were prepared for electron microscopy by fixation either by the Ryter-Kellenberger (16) technique utilizing 2% osmic acid or by immersion in a combination of 2% osmic acid and 2% $KMnO_4$ (3). After fixation, the cells were bathed for 2 hr in 0.5% uranyl acetate (16), dehydrated through graded concentrations of acetone, and embedded either in Araldite (R. P. Cargille Laboratories, Inc., Cedar Grove, N.J.) or in Epon 812 (Shell Oil Co., New York, N.Y.). Sections were cut with a diamond knife on a Porter-Blum model MT-2 ultramicrotome, placed on naked 400-mesh copper grids, stained 30 sec with lead citrate (28), and examined in an RCA EMU-3G or a Siemens Elmiskop 1A electron microscope.

RESULTS

General composition. A comparison of the general composition of cells, spores, and refractile bodies of *B. popilliae* is given in Table 1. The spores and refractile bodies were similar in that they had considerably lower levels of protein, RNA, and DNA than the cells, but the refractile bodies had a considerably higher concentration of PHB and cold trichloroacetic acid-soluble phosphate than did the spores. Also, the spores contained DPA and the refractile bodies did not. The DPA in the spores could not be assayed by the method of Janssen et al. (14) because of interfering materials present in the acid extracts. Its presence and concentration were established by comparing the ultraviolet absorption spectra of the ether extracts prepared by the procedure of Perry and Foster (26) with that of known DPA.

Enzyme composition. Spores of *B. popilliae* did not contain high levels of many of the enzymes unique to, or present in high levels in, spores of some other aerobic bacilli (Table 2). Thus, there was no alanine racemase or glucose dehydrogenase detectable, and the activities of adenosine deaminase, soluble $NADH_2$ oxidase, and pyrophosphatase were lower in spores than in cells. However, of particular interest are the catalase and ribosidase activities in both spores and refractile bodies. No catalase activity was detectable in cells, whereas both spores and refractile bodies had considerable activity, and a fraction of the activity withstood heating at 80

TABLE 1. *Comparison of the composition of cells, spores, and refractile bodies*[a]

Component[b]	Vegetative cells	Spores	Refractile bodies
Protein	47	33	33
RNA	14.0	3.2	2.9
DNA	1.7	0.9	0.6
PHB	4.3	0.6	5.0
Phosphate	1.5	0.6	2.3
DPA	0.0	1.9	0.0

[a] See Materials and Methods for analytical methods. The cells used were harvested from a 24-hr culture in TYG, the spores were from larvae, and the refractile bodies from 14-day cultures in TB broth plus BHB.

[b] Expressed in milligrams per 100 mg (dry weight).

TABLE 2. *Comparison of enzyme activities*[a]

Enzymes	Specific activities[b]		
	Vegetative cells	Refractile bodies	Spores
Group I. High activities in spores of some aerobic bacilli			
Adenosine deaminase	0.23[c]	0.25[c]	0.15[c]
Alanine racemase	0	0	0
Catalase			
Unheated	0[d]	810[d]	210[d]
Heated, 80 C, 10 min	0	160	90
Glucose dehydrogenase	0	0	0
$NADH_2$ oxidase, soluble	0.35	0.08	0.01
Pyrophosphatase			
No Mn^{++}	4.4[c]	0.8[c]	0.5[c]
Plus Mn^{++}	7.8[c]	1.3[c]	1.0[c]
Ribosidase (particulate)	0.34[c]	3.06[c]	2.61[c]
Group II. Catabolic enzymes			
Aldolase	0.38	0.33	0.29
Enolase	1.73	1.28	0.54
Lactic dehydrogenase	1.54	0.53	0.31
Phosphotransacetylase and condensing enzyme	0.003	0	0
Aconitase	0.66	0.15	0
BHB dehydrogenase	0.58	0.76	0.82
Group III. Electron transport			
Diaphorase	0.75	0.28	0.22
$NADH_2$ oxidase, particulate	0.94	0.15	0.04
Cytochrome *c* reductase	0	0	0
Succinic cytochrome *c* reductase	0	0	0

[a] See Materials and Methods for assay methods.

[b] Expressed as change in optical density per minute per milligram of extract protein. Exceptions are indicated with footnotes.

[c] Expressed in micromoles of product per hour per milligram of extract protein.

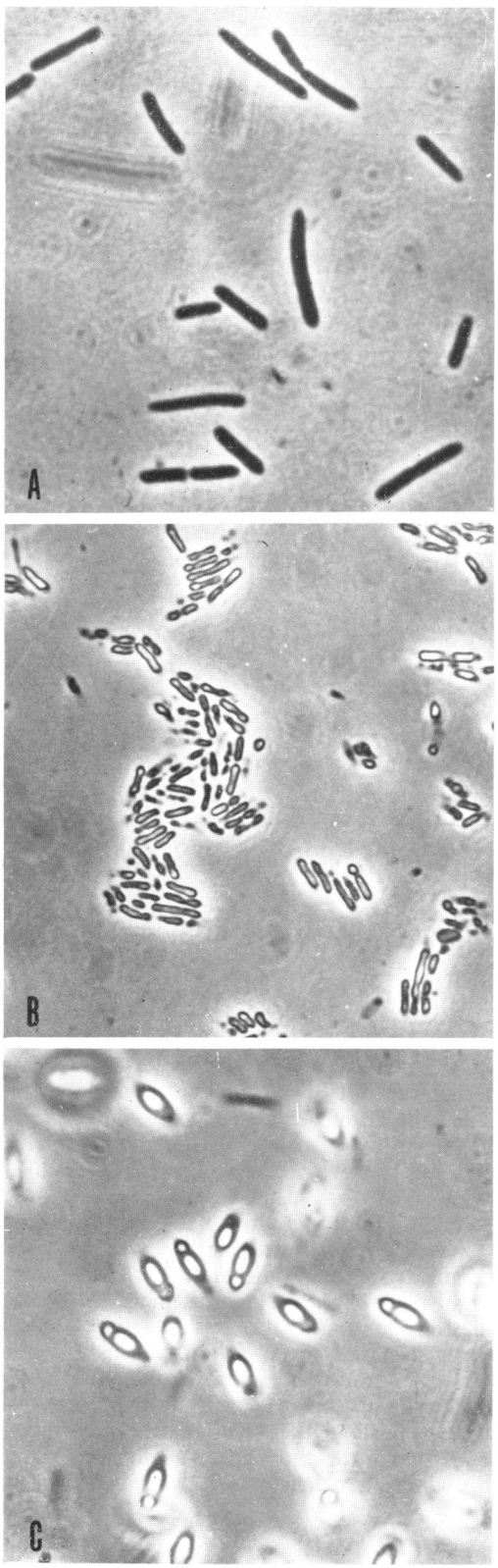
[d] Expressed in microliters of O_2 produced in 1 hr per milligram (dry weight). Calculated from the rate of O_2 released during the first 4 min.

C for 10 min. The catalase was sensitive to 0.01 M cyanide and was completely inactivated by autoclaving at 15 psi for 20 min. The ribosidase activity in the large particles in extracts of refractile bodies and spores was from 8 to 10 times higher than that in supernatant fluids after centrifuging at 3,000 $\times$ *g* for 15 min.

The activities of most of the catabolic and electron transport enzymes assayed were lower in refractile bodies and spores than in cells. However, the activity of aldolase was about equal in all three, and BHB dehydrogenase was lower in cell extracts than in the other two forms. No cytochrome *c* reductase or succinic cytochrome *c* reductase was evident in any of the extracts.

The catalase activity detected in refractile bodies was not present in cells of the same culture age (14 to 21 days) in the TB medium to which 0.1% glucose was added or in the medium with barbituric acid omitted. As reported previously (8), essentially no refractile bodies appeared in the latter cultures. Neither was catalase detectable in cells in TB medium prior to the development of refractility. Catalase was demonstrated in refractile bodies of two strains of *B. popilliae* (B-2309-P-A and B-2309M).

That the catalase activity detected in spores harvested from diseased larvae was truly associated with the spore rather than with the hemolymph of the larvae was indicated by the following facts: (i) after washing the harvested spores six times, there was no further reduction in activity during six more washings, and (ii) more than 40% of the activity in the spores was stable to heating at 80 C for 10 min, whereas the catalase in hemolymph from noninfected larvae was completely inactivated by this treatment.



The catalase in refractile bodies was more sensitive to heat than that in larval spores; only about 20% of the activity was detected after heating as indicated above.

Structure. A comparison of vegetative cells, refractile bodies, and spores of *B. popilliae* by phase-contrast microscopy is shown in Fig. 1. The refractile bodies are smaller than vegetative cells and spores. Though refractile, these bodies do not display the characteristic arrangement of an ellipsoidal spore and a juxtaposed parasporal inclusion encased in a phase-dark sporangium, but they are slender, irregularly shaped, and frequently partitioned.

Comparison of the three cell types at the electron microscopic level is presented in Fig. 2. In contrast to the finely ordered appearance of vegetative cells or spores, the refractile bodies are ghost-like, their relatively empty cytoplasm being characterized by remnants of membrane, possibly of mesosomal origin, and by irregular accretions of electron-dense material. Similar cytoplasmic accretions are observed just prior to sporulation of cells in hemolymph of infected larvae (Black, *unpublished observation*). However, cytological evidence for spores, or even of spore primordia, in refractile bodies is lacking.

DISCUSSION

The spores harvested from infected larvae and the refractile bodies produced in a Tryticase-barbiturate medium are similar, but are distinct from vegetative cells of *B. popilliae* in protein, nucleic acid, and enzyme composition. The protein content of the spores of this species is considerably lower than that observed in spores of *B. megaterium* (37), but the comparative levels of RNA and DNA in spores and cells agree with those observed in *B. cereus* (35). In comparison with cells, the spores and refractile bodies contained high ribosidase and catalase activities, but were generally low in a number of other enzymes assayed. The catalase activity was notable since cells do not produce catalase.

These and previous observations (8) indicate that the refractile bodies are the end result of aborted sporulation. If this is true, the differentiation process becomes abortive at an early stage. Thus, the refractile bodies are considerably higher in PHB and acid-soluble phosphate than the spores, and they contain no DPA. More importantly, they are morphologically and cytologically very dissimilar. However, the electron-dense inclusions observed in refractile

FIG. 1. *Comparison of three cell types by phase-contrast microscopy.* × *3,600.* (*A*) *Vegetative cells of Bacillus popilliae B-2309-P-A.* (*B*) *Refractile bodies produced in TB medium.* (*C*) *Spores of B. popilliae from infected larvae of Popillia japonica.*

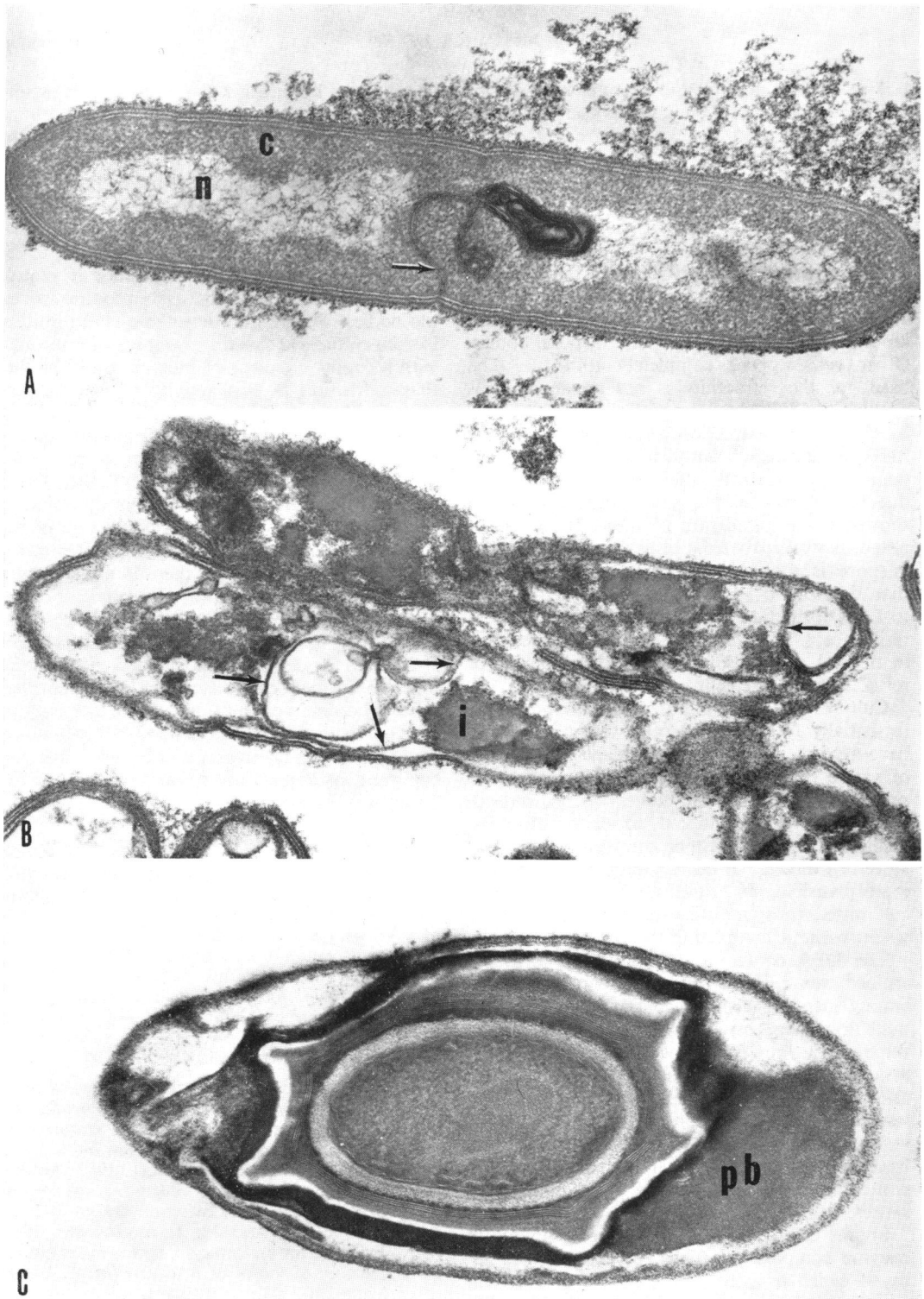


FIG. 2. *Comparison of three cell types of Bacillus popilliae by electron microscopy. (A) Vegetative cell. × 45,000. Note the extensive invagination of the plasma membrane (arrow). This cell is shown in entirety for comparative purposes. The ribosomes in the granular cytoplasm (c) are not resolved at this magnification, and the nucleoplasm (n) has a condensed rather than the more usual stranded appearance. (B) Refractile bodies. × 45,000. The cytoplasm is characterized by membranous remnants (arrows) and by inclusions (i) of electron-dense material resembling that of the parasporal bodies in normal sporangia. (C) Mature spore with sporangium. × 58,280. The black outline delineates the spore proper, comprised of a multilayered integument and a core; the substructure of these has been identified previously (3). Outside the spore and within the sporangial cell wall is a parasporal body (pb). Cells and refractile bodies were fixed in 2% osmic acid and embedded in Epon. Spores were fixed in 2% osmic acid and 2% $KMnO_4$ and were embedded in araldite.*

bodies have a density and texture very similar to those of parasporal bodies, and such inclusions are constantly observed in cells from Japanese beetle larvae just prior to the formation of the spore primordium (Black, *unpublished observations*). Refractile bodies are very difficult to fix, and there may be considerable distortion during this process.

The intensity and distribution of the refractile bodies vary from cell to cell (Fig. 1). Some cells contain both large and small bodies; the latter are similar in size and shape to parasporal bodies. Other cells appear completely refractile. The basis for the refractility is not apparent. It is not due to PHB, since the bodies are not affected by chloroform extraction and since the level of PHB is not significantly higher than that in young vegetative cells. Neither is the refractility due to polysaccharides, since chemical analysis showed no accumulation of these. It should be noted, however, that the basis for the refractility of spores is still not known. It has been established that developing spores become refractile prior to DPA formation and the development of heat resistance (31). Thus, the change(s) occurring in normal sporulation and the development of refractility in these cells could be similar.

Cultures in which the highest percentage (essentially 100%) of the cells were observed to have refractility contained a higher percentage of viable cells than similar cultures with lower percentages of refractile bodies (8). If, indeed, these cells are committed to sporulation, the viable fraction must either complete the process or return directly to cell division. The former is more plausible, since it is not believed that cells committed to sporulation can undergo cell division without completing the sporulation cycle.

The DPA content of *B. popilliae* spores examined was quite low. Although this may vary among lots, the average concentration must be quite low since no DPA could be detected in three other lots of spores by the colorimetric assay procedure of Janssen et al. (14). The low DPA content may be correlated with the relatively low heat resistance (29) and with the low germinability of these spores (33). Previous studies (3, 7) have demonstrated a correlation between DPA content and these characteristics in aerobic spores.

Spores of *B. popilliae* appear to be unique in enzyme composition. Thus, of six enzymes assayed which have been reported to be enriched in spores of some aerobic bacilli (2, 13), only ribosidase was found to have a higher activity in extracts of spores than in cell extracts. The failure to detect alanine racemase in either cells or spores is of much interest. Nutritional studies (36) have demonstrated that this organism does not require exogenous alanine, so it must synthesize both the D and L isomers when D-alanine is present in the cell wall.

The presence of catalase in spores of this species is of particular interest, since no catalase is detectable in cells grown in laboratory media. The production of this enzyme appears to be associated with morphogenesis, since it is produced at the same time that cells produce refractile bodies. There is no detectable multiplication in such cultures at this time, so it is not associated with secondary growth of mutant cells. The fact that a fraction of this activity is heat-resistant also indicates association with sporogenesis. Lawrence and Halvorson (19) demonstrated the presence of catalase in spores of *B. terminalis* all of which which was stable to 80 C for 10 min, but the total activity was much less than that of corresponding cells. It is possible that all of the catalase in mature spores of *B. popilliae* is stable to 80 C for 10 min, since there is no assurance that the washing procedure eliminated all of the heat-sensitive catalase of the hemolymph from which these spores were harvested. *B. popilliae* B-2309 M undergoing sporulation on a solid medium formed catalase at the time morphogenesis became apparent, and all of the enzyme was stable to this heat treatment (Costilow, *unpublished data*). Therefore, it is believed that this enzyme constitutes a good marker indicating the onset of sporulation.

ACKNOWLEDGMENTS

This investigation was supported by contracts with the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill.

The cooperation of the personnel of the Division is greatly appreciated. We are particularly grateful to Grant St. Julian for producing and harvesting the spores from larvae of the Japanese beetle.

LITERATURE CITED

1. ANFINSEN, C. B. 1955. Aconitase from pig heart muscle, p. 695–698. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.
2. BACH, J. A., AND H. L. SADOFF. 1962. Aerobic sporulating bacteria. I. Glucose dehydrogenase of *Bacillus cereus*. J. Bacteriol. **83**:699–707.
3. BLACK, S. H., AND M. I. ARREDONDO. 1966. Evidence for an intracytoplasmic membrane in the core of spores of *Bacillus popilliae*. Experientia **22**:77–78.
4. BLACK, S. H., T. HASHIMOTO, AND P. GERHARDT. 1960. Calcium reversal of the heat susceptibility and dipicolinic deficiency of spores formed "endotrophically" in water. Can. J. Microbiol. **6**:213–224.

5. BUCHER, T. 1955. Enolase from brewer's yeast, p. 427–434. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc. New York.
6. BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315–323.
7. CHURCH, B. D., AND H. O. HALVORSON. 1959. Dependence of the heat resistance of bacterial endospores on their dipicolinic acid content. Nature **183**:124–125.
8. COSTILOW, R. N., C. J. SYLVESTER, AND R. E. PEPPER. 1966. Production and stabilization of cells of *Bacillus popilliae* and *Bacillus lentimorbus*. Appl. Microbiol. **14**:161–169.
9. DELAFIELD, F. P., K. COOKSEY, AND M. DOUDOROFF. 1965. β-Hydroxybutyrate dehydrogenase and dimer esterase of *Pseudomonas lemoigni*. J. Biol. Chem. **240**:4023–4028.
10. DOI, R. H., AND H. HALVORSON. 1961. Comparison of electron transport system in vegetative cells and spores of *Bacillus cereus*. J. Bacteriol. **81**:51–58.
11. DOWLER, W. M., P. D. SHAW, AND D. GOTTLIEB. 1963. Terminal oxidation in cell-free extracts of fungi. J. Bacteriol. **86**:9–17.
12. FISKE, C. H., AND Y. SUBBAROW. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. **66**:375–400.
13. HALVORSON, H. 1962. Physiology of sporulation, p. 223–264. *In* I. C. Gunsalus and R. Y. Stanier [ed.], The bacteria, vol. 4. Academic Press, Inc., New York.
14. JANSSEN, F. W., A. J. LUND, AND L. E. ANDERSON. 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science **127**:26–27.
15. JOHNSON, M. J. 1941. Isolation and properties of a pure yeast polypeptidase. J. Biol. Chem. **137**: 575–586.
16. KELLENBERGER, E., A. RYTER, AND J. SECHAUD. 1958. Electron microscope study of DNA-containing plasms. J. Biophys. Biochem. Cytol. **4**:671–678.
17. KORNBERG, A. 1955. Lactic dehydrogenase of muscle, p. 441–443. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.
18. LAW, J. H., AND R. A. SLEPECKY. 1961. Assay of poly-β-hydroxybutric acid. J. Bacteriol. **82**: 33–36.
19. LAWRENCE, N. L., AND H. O. HALVORSON. 1954. Studies on the spores of aerobic bacteria. IV. A heat resistant catalase from spores of *Bacillus terminalis*. J. Bacteriol. **68**:334–337.
20. LEVINSON, H. S., J. D. SLOAN, JR., AND M. T. HYATT. 1958. Pyrophosphatase activity of *Bacillus megaterium* spore and vegetative cell extracts. J. Bacteriol. **75**:291–299.
21. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
22. MEJBAUM, W. 1939. Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. Z. Physiol. Chem. **258**:117–120.
23. OCHOA, S. 1955. Crystalline condensing enzyme from pig heart, p. 685–694. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.
24. PEPPER, R. E., AND R. N. COSTILOW. 1964. Glucose catabolism by *Bacillus popilliae* and *Bacillus lentimorbus*. J. Bacteriol. **87**:303–310.
25. PEPPER, R. E., AND R. N. COSTILOW. 1965. Electron transport in *Bacillus popilliae*. J. Bacteriol. **89**:271–276.
26. PERRY, J. J., AND J. W. FOSTER. 1955. Studies on the biosynthesis of dipicolinic acid in spores of Bacillus cereus var. mycoides. J. Bacteriol. **69**: 337–346.
27. POWELL, J. F., AND J. R. HUNTER. 1956. Adenosine deaminase and ribosidase in spores of *Bacillus cereus*. Biochem. J. **62**:381–387.
28. REYNOLDS, E. S. 1963. The use of lead citrate at high *p*H as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**:208–212.
29. RHODES, R. A. 1965. Symposium on microbial insecticides. II. Milky disease of the Japanese beetle. Bacteriol. Rev. **29**:373–381.
30. RHODES, R. A., M. S. ROTH, AND G. R. HRUBANT. 1965. Sporulation of *Bacillus popilliae* on solid media. Can. J. Microbiol. **11**:779–783.
31. SADOFF, H. 1966. The effect of gluconate in promoting sporulation in *Bacillus cereus*. Biochem. Biophys. Res. Commun. **24**:691–695.
32. SIBLEY, J. A., AND A. L. LEHNINGER. 1949. Aldolase determinations. J. Biol. Chem. **177**:859–862.
33. SPLITTSTOESSER, D. F., AND K. H. STEINKRAUS. 1962. Factors influencing germination and outgrowth of *Bacillus popilliae* spores. I. Effect of potassium ions. J. Bacteriol. **84**:278–282.
34. STEWART, B. T., AND H. O. HALVORSON. 1953. Studies on the spores of aerobic bacteria. I. The occurrence of alanine racemase. J. Bacteriol. **65**:160–166.
35. STUY, J. H. 1958. The nucleic acids of *Bacillus cereus*. J. Bacteriol. **76**:179–184.
36. SYLVESTER, C. J., AND R. N. COSTILOW. 1964. Nutritional requirements of *Bacillus popilliae*. J. Bacteriol. **87**:114–119.
37. TINELLI, R. 1955. Étude de la biochimie de la sporulation chez *Bacilles megaterium*. I. Composition des spores obtenues par carence des différents substrats carbonés. Ann. Inst. Pasteur **88**:212–226.