GROWTH AND SPORULATION CHARACTERISTICS OF AN ORGANIC SULFUR-REQUIRING AUXOTROPH OF BACILLUS CEREUS

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Received for publication 12 April 1963

ABSTRACT

LUNDGREN, D. G. (Syracuse University, Syracuse, N.Y.) and K. F. BOTT. Growth and sporulation characteristics of an organic sulfur-requiring auxotroph of Bacillus cereus. J. Bacteriol. 86:462–472. 1963.—This paper reports investigations of several aspects of growth and sporulation of an organic sulfur-requiring auxotroph of Bacillus cereus ATCC 4342. The wild type and B. cereus T were also studied for comparative purposes. Growth of the mutant on minimal medium plus methionine was normal, but sporulation was completely inhibited. Some reaction involved in vegetative-cell maturity was probably blocked at a point just prior to the “triggering” of sporulation, since abnormally large amounts of poly-β-hydroxybutyric acid (PHB) were formed. Growth of the mutant in the presence of cystine or cysteine was also accompanied by the build-up of large amounts of PHB, but some endospores were formed (approximately 5% by 72 hr). Results of dipicolinic acid (DPA), calcium, and heat-resistance studies revealed that the few spores formed by the auxotrophic mutant when grown on cysteine were somewhat below wild-type strains in their development of heat resistance, and considerably lower in content of Ca and DPA. This was not the case with spores formed in cells grown on cystine; heat resistance compared favorably with wild-type spores, but the Ca and DPA levels were lower.

An earlier report from this laboratory described a procedure for the isolation of ultraviolet-induced organic sulfur-requiring auxotrophic mutants of Bacillus cereus (Bott and Lundgren, 1962). Subsequent investigations have centered around the use of one of these mutants as a tool in the study of various sporulation mechanisms, especially the phenomenon of spore heat and radiation resistance.

A foundation for this work was provided by the findings of Vinter (1956, 1957, 1960, 1961). Although his studies did not employ auxotrophic mutants, Vinter (1956, 1960) reported that additions to sporulating B. cereus and B. megaterium of 10^{-3} or 10^{-4} M cysteine caused a noticeable inhibition in the normal sporulation process. The 10^{-4} M concentration enabled formation of an abnormally low percentage of spores which could be distinguished from normal spores by a more rounded appearance and a lesser degree of light refractility. The effect of cyst(e)ine was also shown to affect only the sporulation process and not vegetative-cell development (Vinter, 1957). Its action was proposed to result from the toxic presence of H_{2}S, a breakdown product of cyst(e)ine.

Through the use of isotopic studies, Vinter (1961, 1962) was able to separate the development of spore resistance into two stages. The first stage, development of radioresistance, had to be completed in a normal manner before the second stage, development of heat resistance, progressed.

This paper reports the initial investigations designed to characterize the growth and sporulation patterns of the sulfur-requiring auxotroph on three sulfur-containing amino acids. The latter phases of the research, which concentrated upon the radioresistance of cells and spores under these same conditions, will be reported in a separate publication.

MATERIALS AND METHODS

Organisms. The wild-type (parent) organism used in this investigation was B. cereus ATCC 4342. In addition, an organic sulfur-requiring auxotroph of the parent, isolated after ultraviolet irradiation and designated as C-1, was also studied. The procedure for isolation of this mutant has been described (Bott and Lundgren, 1962).
The mutant organism required for growth the addition of cysteine, cystine, methionine, homocysteine, homocysteine, or cystathionine to the regular minimal medium. The growth pattern of the mutant on complex media was indistinguishable from the wild type. A third organism, used as a reference organism, was \( B. \text{ cereus} \) T, obtained from H. O. Halvorson, University of Illinois.

**Medium and culture maintenance.** The basic minimal medium employed in these studies was the glucose-glutamate-glycine-salts (GGGS) medium of Lundgren and Beskid (1960), with the exception that in the salts solution used here 25 mg/liter of CaCl₂ were substituted for CaHPO₄.

For mutant C-1, the desired amino acid concentration of filter-sterilized aqueous solutions of L-methionine, L-cysteine, or L-cysteine·HCl was added aseptically to the minimal medium after autoclaving, from stock concentrated solutions. Cystine stocks were acidified with HCl to facilitate solution and were filter-sterilized with an ultrafine porosity Pyrex fritted-glass filter.

Maintenance of cultures and preparation of the standard spore inocula were as described by Lundgren and Beskid (1960). The absolute requirement of the mutant for an added organic sulfur amino acid was verified prior to every experiment.

In experiments utilizing mutant C-1, 50 μg/ml of cysteine or methionine and 40 μg/ml of cystine were designated as optimal concentrations of these amino acids. The choice was based upon analysis of shaken 250-ml Erlenmeyer flasks containing 12 different concentrations (ranging from 1 to 1,000 μg/ml) of each amino acid. Optimal growth was chosen as that concentration giving maximal turbidity by 72 hr.

**Inoculum.** A synchronized inoculum, prepared in shaken flasks at 37 C, was employed in all experiments. Its preparation involved the use of heat-activated (65 C, 15 min) spores and three subsequent cell transfers into fresh media. Transfers were made after 5, 4, and 3 hr of culture development. The regular GGGS medium was supplemented with 0.2% yeast extract for the first stage (plus the optimal concentration of sulfur-containing amino acid in each case for mutant C-1). Amounts of 100 ml of this inoculum were conventionally added to each large culture vessel containing 1.5 liters of GGGS medium.

**Culture apparatus.** Large-volume cultures were routinely used to harvest periodic samples of cells grown at 37 C. The culture vessels employed were made from 2-liter glass solution bottles, and have been previously described by Cooney and Lundgren (1962). However, in the present studies two fritted-glass spargers (Corning \# 3953, EC, Corning Glass Works, Corning, N.Y.) were employed in each culture vessel to provide maximal aeration (1,600 cc of air per 1.5 liters per min, as measured with a F and P tri-flat flowrator (Fisher & Porter Co., Hatboro, Pa.)).

Hydrogen sulfide evolution from the aerators was detected by connecting a long glass tube from the exhaust port to a 500-ml Erlenmeyer flask containing 400 ml of 0.1% acidified aqueous lead acetate, and bubbling all exit air through it. The development of a black precipitate (PbS) was considered indicative of H₂S production.

**Culture growth.** Culture growth studies of \( B. \text{ cereus} \) ATCC 4342, \( B. \text{ cereus} \) T, and mutant C-1 were made by using the culture apparatus previously described. Mutant C-1 was grown in GGGS medium plus L-cysteine or L-methionine at concentrations of 0, 20, 50, and 80 μg/ml and in L-cystine at 0, 20, 40, and 60 μg/ml. The inoculum for all analyses of mutant C-1 was prepared by using the optimal concentration of the test amino acid. No concentration correction was made for the unutilized amino acid present in the inoculum fluid. The sampling interval for each study was varied to cause more frequent determinations during the log and early stationary phases of growth, with less frequent determinations made once the growth plateau had been reached.

At each sampling time, 15 to 20 ml of cell suspension were withdrawn into sterile screw-capped test tubes and stored at −20 C until used for cell viability, dry weight, and dipicolinic acid (DPA) determinations. In addition, a second cell sample was collected for population-growth studies, as well as for microscopic examinations. Growth was measured turbidimetrically with a Klett-Summerson photoelectric colorimeter and a blue filter (420 mполки). The pH fluctuations of the culture were also followed by using a conventional Beckman-type pH system.

**Microscopy.** Microscopic observations of collected cells were made at the time of each cell harvest. Wet-cell mounts were examined by phase-contrast microscopy at a magnification of 1200 × with a Zeiss (model GFL) binocular microscope (Brinkmann Instruments, Inc., Great Neck, N.Y.).
Spore and fat-stained smears were viewed by regular light microscopy at a magnification of 1200 × with the same microscope but without the phase optics. Spores were stained by the method of Schaeffer and Fulton (1933). Fat stains were performed by the method of Burdon (1946). Photomicrographs were taken with a Leitz Micro Ibsö 35-mm camera mounted on the Zeiss model GFL microscope.

Viability and DPA analyses. All samples for a particular experiment, or (with mutant C-1) one complete sample series for a single amino acid concentration, were assayed at the same time to avoid any possible inconsistency in experimentation.

Viable counts were made on thawed samples by using the pour plate technique. All counts were made in triplicate, with saline as diluent and Brain Heart Infusion Agar as the nutritive environment.

After the portion had been removed from the thawed sample for cell viability counts, 12 ml of the cell suspension were centrifuged at 3,000 × g, and the cell pellet was washed twice with sterile distilled water and resuspended in an equal volume of distilled water. Portions (1 ml) of the washed-cell suspension were added to duplicate tared planchets, and dried for 48 hr at 105 °C prior to dry weight determinations. Two 4-ml samples were removed from the washed suspension and analyzed for DPA, according to the method of Janssen, Lund, and Anderson (1958). DPA from K and K Laboratories, Jamaica, N.Y., was used in preparation of the standard curve.

Sonic cleaning and chemical analysis of spores. At the end of each culture cycle (maximal production of endospores), cellular material and spores were collected by centrifugation. The cell pellet, in a 50-ml polypropylene centrifuge tube, was suspended in 35 ml of sterile distilled water and sonically treated for 8 min at an intensity equivalent to a dial setting of “7” with a 20-kc Branson Sonifier (Heat Systems Co., Great Neck, N.Y.).

Raymond Testa (unpublished data) in this laboratory previously showed that 30 min of sonic treatment at this intensity with glass beads had no noticeable microscopic effect on B. cereus spores. During sonic treatment, the polypropylene tube was suspended in an ice bath, and immediately after sonic treatment spores were separated from the vegetative-cell debris by differential centrifugation and were washed five times with sterile distilled water. Spore cleanliness was judged by using phase optics. Cleaned spores were stored at 5 °C until further use (which in no case was longer than 21 days).

The DPA analysis was performed as previously described. Calcium determinations were made by using flame photometry (Coleman Instruments, Inc., Maywood, Ill.). The procedure employed was essentially the same as that outlined by Coleman Instruments, Inc. (1958). The exception to the outlined procedure was the addition of 0.15 meq of Na to each sample in the 27-ml volumetric flask. This stabilized the interference of small quantities of sodium in the spores (Cooney, 1961). The wet-ashing procedure described by Lundgren and Cooney (1962) was employed in preparation of spores for this analysis.

Heat-resistance studies. Heat resistance was determined by testing spore viability after 30 min of heat treatment at 60, 70, and 80 °C. Portions (1 ml) of the spore suspension (in saline) were added to each of four tubes containing 9 ml of saline. One tube (control) was kept in an ice bath until the other tubes were heated at 60, 70, and 80 °C, respectively, in constant-temperature water baths. After 30 min had elapsed, all tubes were immediately plunged into an ice bath and kept until each suspension was plated (in triplicate) on Brain Heart Infusion Agar. All tests were done in duplicate for spores and for 70-hr vegetative cells of mutant C-1 grown on methionine.

RESULTS

Growth studies of B. cereus T and B. cereus wild type. Figures 1 and 2 illustrate the cultural growth characteristics of the two reference organisms, B. cereus T and wild-type B. cereus. No major cultural differences were noted between the two strains. However, B. cereus T completed its cultural cycle in slightly less time, indicated by the more rapid attainment of 90% endospores. This may indicate that a higher degree of cultural synchrony was obtained with this strain. Viable-cell count data (Fig. 2) indicate that in both strains the increase in optical density was directly related to an increase in cell number, with approximately 6 × 10⁶ cells/ml at maximal turbidity.

Figure 1 also shows the pH changes of the medium during growth and sporulation. These findings were found to be in good agreement with published reports (Black, Hashimoto and Gerhardt, 1960; Cooney, 1961).
Results of DPA analyses of these cells (Fig. 1) revealed that the wild-type B. cereus began synthesis of DPA earlier than did B. cereus T and contained a higher level in mature spores.

Microscopic observations of these two strains showed no major morphological differences at any stage of development. Hashimoto, Black, and Gerhardt (1960) have shown the stages of sporogenesis in B. cereus T recognizable by dark phase-contrast microscopy.

Photomicrographs of the wild type are shown for comparative purposes (Fig. 3) to illustrate the production of the sudanophilic inclusions of poly-β-hydroxybutyrate (PHB) in vegetative cells. Fat-stained smears showed all inclusions to be intensely stained with Sudan black B. The stage of sporogenesis called the "granular stage" is characterized by the production of PHB granules which tend to diminish as later stages of the cultural growth cycle occur.

For use in comparing these two reference strains with the general characteristics of mutant C-1 (to be described later), several features should be emphasized. The most significant feature of the general culture patterns was that the culture cycle reached the mature spore stage by 24 hr, with over 95% of the cells producing spores. The sudanophilic inclusions never occupied more than half of the cell volume, and a high degree of culture synchrony was maintained throughout the cycle. No hydrogen sulfide was detectable during the growth of either of these strains.

Growth studies of mutant C-1. Vegetative growth and sporulation patterns of mutant C-1 when grown in the presence of methionine, cysteine, or cystine were quite different from the wild-type organism. Sporulation was completely inhibited...
Observations showed that these mutant cells contained a small amount of lipid material at maximal turbidity, and the polymer level gradually decreased during the rest of the incubation period.

Culture characteristics on methionine. Figures 4 and 5 illustrate the characteristic growth responses of mutant C-1 to different levels of methionine. Viable-cell counts failed to parallel the continued rise in turbidity during culture development. The rise observed was undoubtedly related to the accumulation of PHB in the cells. The pH changes in the medium were approximately the same regardless of the concentration of methionine added to the minimal medium. The medium pH rose to a maximum of pH 8 by 18 hr, then gradually declined, and leveled off slightly below pH 6. This pH pattern was, however, considerably different from the pH trends observed in the wild-type culture (Fig. 1). Culture synchrony in the presence of methionine was obtained only during the initial log phase of development, and once cells accumulated abnormal amounts of PHB (approximately 15 to

![Photomicrographs of Sudan black B-stained smears of wild-type Bacillus cereus at different stages of development: (a) 8-hr cells at beginning of PHB build-up; (b) 12-hr cells containing maximal PHB.](image)

In methionine-grown cells, and approximately 5% spores were noted in cyst(e)ine-grown cells but only after extending the culture time to 72 hr. All three sulfur sources stimulated the production of abnormally large amounts of poly-β-hydroxybutyric acid. Results of DPA analyses revealed no detectable DPA during the early growth cycle and only trace levels at the 70-hr period. The latter levels were at or slightly above the accepted experimental limit of the test.

With all three sulfur sources, growth was highly flocculent and settled out rapidly as soon as the aeration was stopped. Microscopic observations showed that this feature was probably due to abnormal cell clumping and chain formation.

In all experiments, the control growth medium (which contained only the sulfur amino acid introduced with the inoculum) increased in turbidity to slightly less than 200 Klett units and then remained at that level. Microscopic ob-
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18 hr) synchrony was lost. At this time, young vegetative cells, lacking large inclusions, were frequently observed. By the last 12 to 14 hr of culture incubation all cells contained maximal PHB.

Fluctuations in the viable-cell counts probably resulted from periodic lysis of some cells which upset regulated cell growth and the loss of culture synchrony. The gradual buildup of PHB in cells and polymer in the medium as the result of cell lysis could have accounted for the steady increase in turbidity. This prolonged production of PHB made the cells appear very "mottled" when stained by spore stains, and larger in diameter than the wild-type cells. No spores were formed when mutant C-1 was grown on methionine. This finding was supported by spore stains as well as heat-sensitivity experiments. No hydrogen sulfide was detected when this amino acid supported growth, and no DPA was detected in any of the cells analyzed.

Morphological differences were very slight when mutant C-1 was grown on the three different concentrations of methionine. Figure 6a illustrates the extent of buildup of PHB as observed by phase microscopy. The lightly refractile bodies are granules of PHB. Some of these bodies certainly resemble spores, but results of both staining and chemical tests showed them to be butyrate. Photomicrographs of spore-stained smears showed that this polymer occupied a very large percentage of the total cell volume and was frequently seen as two or three equal-size inclusions within the cell (Fig. 6b). Fat

FIG. 6. Photomicrographs showing polymer in mutant C-1 grown on methionine, as revealed by staining and phase optics. (a) Phase-contrast view of 69-hr-old cells; slightly refractile inclusions represent PHB. (b) Spore-stained smear of 69-hr-old cells; cells appear very "mottled" where the polymer failed to stain; some cells have three to four vacuoles. (c) Fat-stained smear of 12-hr-old cells; vacuole as shown in b was stained with Sudan black B; by 69 hr, cells appeared "stuffed" with polymer.
stains of similar cells frequently "distorted" this picture by showing one large inclusion, resulting from excess Sudan black B stain taken up by this material (Fig. 6c).

Cultural characteristics of cysteine and cystine. The characteristic culture growth patterns of mutant C-1 grown on cysteine are illustrated in Fig. 7 and 8. Culture turbidity did not follow cell viability when this amino acid was used. The viability and turbidity curves for cystine-grown cells were similar and are not shown. In each case, after an initial parallel development of both curves, the viability counts reflected a periodic fluctuation in cell populations, probably resulting from culture lysis and reutilization of liberated constituents. The culture turbidity curves rose continuously over the entire culture time. The different levels of these amino acids used to culture the organism had some effect upon the organisms as indicated by these curves. As was the case with methionine-grown cells, the increase in optical density of these cultures beyond the period of 15 to 18 hr appeared to be the result of increased PHB production.

Changes in the pH of the medium during growth of the mutant on cysteine are also shown (Fig. 7). Again some variation was noted from normal pH changes (Fig. 1), and differences in culture response to the various concentrations of the amino acid were detected. The middle concentration of cyst(e)ine caused a pH dip after 24 hr which rose to slightly above pH 8 during the remainder of the culture cycle; this type of pH response is not uncommon (Halvorson, 1957). However, the low concentration of cysteine resulted in an initial rise of the pH in the medium and a decline after 24 hr, while the medium containing cystine at the same concentration maintained a level pH until 30 hr and then rose. The highest concentration of cysteine caused an initial dip in the pH of the medium to 5.8 which then began to rise to a maximum above pH 8 around 48 hr. With the high concentration of cystine, no final rise was noted after the early drop.

Mutant C-1 when grown on either of these sulfur-containing amino acids formed a few spores by 70 hr. All spores formed failed to stain intensely with malachite green, and also were less light-refractile than wild-type spores. The
maximal amount of spores formed in the presence of these amino acids was approximately 5% and occurred at a cysteine level of 80 µg/ml and a cystine level of 40 µg/ml. All spores formed had similar morphological characteristics, regardless of the level of cyst(e)ine.

Morphologically, no gross differences were observed in vegetative cells grown on cyst(e)ine, although, as in the case of methionine, the PHB was observed to occupy a slightly larger portion of the cell when higher concentrations of the sulfur additions were used. The PHB material in cells grown on cyst(e)ine was frequently contained in one or two large granules as opposed to the larger number of smaller granules observed with methionine-grown cells.

The only DPA detectable in these cells was a "trace" observed at the last sampling period for each concentration. Growth of mutant C-1 when cyst(e)ine was present at each of the three concentrations resulted in the production of considerable amounts of H2S. The lead acetate trap connected to each fermentor was "jet black" by 15 hr. As culture growth progressed, the culture grown with the highest concentration of amino acid produced more H2S and at an earlier time, usually by 10 to 12 hr. The continued trapping of exhaust air gradually changed the color in these traps so that by 70 hr all were gray in color.

Analysis of sonically cleaned spores. In the past, the enzyme lysozyme was used to clean spores. Although several strains of *B. cereus* are known to be resistant to lysozyme, our strain (*B. cereus* 4342) is normally susceptible to this enzyme (Cooney, 1961; Cooney and Lundgren, 1962). However, in the present study lysozyme did not lyse the mutant vegetative cells, so sonic rupture was used for cleaning spores.

DPA, calcium, and heat resistance. The results of DPA, calcium, and heat-resistance analyses of spores are shown in Table 1. Methionine-grown cells were also analyzed, and these results are recorded for comparative purposes. The 70-hr-old vegetative cells were used, since no spores were noted in the mutant grown with methionine.

Only slight differences were noted between the heat resistance of spores formed in cystine and those of the control strains, although the cystine-formed spores had considerably lower levels of DPA and Ca (Table 1). On the other hand, spores formed in the presence of cysteine had somewhat more DPA and Ca than the

<table>
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<tr>
<th>Prepn*</th>
<th>DPA†</th>
<th>Ca†</th>
<th>Survivors (%) after 30 min at 60 C</th>
<th>70 C</th>
<th>80 C</th>
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<tr>
<td>Wild-type <em>B. cereus</em> spores</td>
<td>106.00</td>
<td>7.60</td>
<td>90.073</td>
<td>048.0</td>
<td>90.073</td>
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<tr>
<td><em>B. cereus</em> T spores</td>
<td>95.50</td>
<td>7.16</td>
<td>99.572</td>
<td>541.0</td>
<td>99.572</td>
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<td>Spores (20 µg/ml of Ci)</td>
<td>5.22</td>
<td>1.06</td>
<td>90.068</td>
<td>648.3</td>
<td>90.068</td>
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<td>Spores (40 µg/ml of Ci)</td>
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<td>100.068</td>
<td>38.8</td>
<td>100.068</td>
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<td>Spores (60 µg/ml of Ci)</td>
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<td>Spores (20 µg/ml of Ce)</td>
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<td>Spores (80 µg/ml of Ce)</td>
<td>15.75</td>
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<td>59.737</td>
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* Ci = cystine; Ce = cysteine; Met = methionine.
† Results expressed as µg per mg of cells.

Cystine spores but were more heat-sensitive. Methionine-grown cells contained no DPA and were not very heat-resistant.

**DISCUSSION**

Culture growth patterns of this mutant when grown on any of the three amino acids were somewhat different, reflecting a difference in the metabolism of these three sulfur-containing compounds. Sporulation in the mutant was completely blocked by methionine and strongly inhibited by cystine and cysteine. The few spores formed in the presence of cyst(e)ine looked abnormal under phase optics, appearing roundish in structure and having less refractility; also, the spores were not intensely stained with malachite green. This spore appearance seemed to correspond to Vinter's (1959) descriptions of spores formed when "cystine inhibition" had been reversed by additions of glucose.

The formation and storage of abnormally large amounts of poly-β-hydroxybutyric acid was associated with the prolonged vegetative state of the mutants. The polymer was positively identified as PHB by Alper, Marchessault, and Lundgren (1962), using the characteristic PHB X-ray diffraction pattern of dried whole cells as a
criterion. The abnormal buildup of PHB might be explained in at least two ways. Either the process of sporulation was inhibited by the presence of these sulfur compounds at a point just prior to where cells normally are “triggered” to utilize their endogenous reserves in spore formation, or perhaps some genetic block prevented the development of spores. If the latter example were true, the production of a small percentage of spores in cyst(e)ine-grown cells could be explained as genetic revertants which had either spontaneously “back mutated” to the spore-forming prototroph, or the mutants were supplied the necessary nutrient(s) by cell lytic products to overcome the block.

Several workers have proposed that the beginning of sporulation is characterized by the induction of an enzyme system which facilitates the utilization of intermediates (acetate) previously excreted into the medium (Krishna Murty and Halvorson, 1958; Gollakota and Halvorson, 1959, 1960; Hanson, Srinivasan, and Halvorson, 1963). If an enzyme such as this were blocked in mutant C-1, sporulation would be inhibited, leaving a type of “vegetative-cell state” and possibly explaining the large accumulation of PHB. Grelet (1957) suggested that two sets of conditions must exist for sporulation in B. megaterium: (i) those conditions which cause vegetative growth to cease and promote spore formation, and (ii) those conditions necessary for development of the mature spore. If this were the case in B. cereus, we might propose that growth of mutant C-1 on the sulfur-containing amino acids resulted in an interference of those conditions promoting spore formation, for vegetative growth had ceased, but spore formation was inhibited. This too could account for large accumulations of PHB.

Bressler and Wakil (1962) proposed that fatty acid synthesis in pigeon liver is stimulated by sulfhydryl-containing compounds. We might also propose that large amounts of PHB acid were built up in these bacteria as a result of the influence of an excess of these or related stimulatory agents.

Vinter (1959) proposed that cyst(e)ine inhibition resulted from hydrogen sulfide, a product of cyst(e)ine degradation, poisoning some oxidative enzyme system such as cytochromes. It is possible that a comparable phenomenon might be present here, since H2S was liberated from cultures grown on cyst(e)ine. This hypothesis, however, offers no explanation for the complete inhibition observed with methionine, since a detectable level of H2S did not accompany the metabolism of this amino acid.

No chemical analyses of the medium were carried out. Consequently, the pH fluctuations in the medium, supporting cells grown on cyst(e)ine, can only be assumed to have resulted from the liberation of metabolic intermediates and cell debris into the medium. However, it is possible that these atypical pH fluctuations represented a reflection of genetically altered cell metabolism in the mutant which was expressed in the form of preferential selection of certain cations in the medium. The pH changes of the media may have been stimulated by the presence of a particular concentration of amino acid.

Changes in the pH of the medium similar to this are not uncommon in studies of sporulation. Halvorson (1957) has shown that the pH of the medium supporting growth of B. cereus characterized dip just before sporulation, then as reutilization of acid intermediates occurred the pH rose. Failure to observe this initial dip in pH of the media in our B. cereus control cultures is believed to be a consequence of the rapid aeration rate and buffering capacity of the medium. This feature was also noted by Cooney (1961). Cyst(e)ine is known to be broken down by bacilli into H2S, pyruvic acid, and NH3 (O’Connor and Halvorson, 1961). Excretion and reutilization of either of the latter two compounds at various times during the growth cycle could conceivably induce fluctuations in pH of the growth medium, although the minimal medium was well buffered.

Except for the first 15 to 18 hr of culture development, the viable-cell counts of mutant C-1 did not parallel the steady rise in culture turbidity. Good correlation between growth and turbidity was noted throughout the culture cycle of the normal B. cereus strains. The steady increase in culture turbidity for mutant cultures, beyond the time where viability leveled off, was believed to be associated with the synthesis of PHB. Such an occurrence has previously been noted by Schlegel, Gottschalk, and von Bartha (1961) in studies of Hydrogenomonas. Fluctuations in cell viability were attributed to periodic lysis and recycling of a portion of the population. Such fluctuations can also explain the noticeable lack of synchrony in the later hours of the mutant growth cycles; good culture
synchrony was maintained in *B. cereus* T and *B. cereus* wild type.

DPA was not detected in significant quantities in any of the cell samples harvested at different times during the growth cycle, except at the later hours for wild type and *B. cereus* T. Even though DPA would not be expected in cultures which lacked spores (Foster, 1959), the inability to detect it during the late hours in cells grown with cyst(e)ine was undoubtedly due to the overwhelming abundance of nonsporulating cells in the culture, and the unusually low concentration present in those spores formed.

The calcium levels of mutant C-1 spores were also lower than spores of the wild-type strains. Since calcium and DPA have been directly correlated with thermoresistance of the bacterial spore (Foster, 1959; Vinter, 1960, 1961) and the spores formed by mutant C-1 grown with cyst(e)ine were observed to have low concentrations of both, it is not difficult to explain the lower heat resistance of cysteine-formed spores. It is, however, odd, and at present unexplainable, why the spores of cystine-grown cells were found to have a heat resistance very similar to that of the wild-type spores. Perhaps the presence of cystine in these cultures has contributed to increased heat resistance, in a manner analogous to that by which cysteic acid is proposed to aid in thermoresistance of some thermophiles (Sobotka and Luisada-Opper, 1957).

**ACKNOWLEDGMENT**

This investigation was supported by contract AT 30-1-2038 of the U.S. Atomic Energy Commission.

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