Poly-β-Hydroxybutyrate Metabolism During Growth and Sporulation of *Clostridium botulinum*

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The granules observed in the cytoplasm of cells of sporogenic and asporogenic strains of *Clostridium botulinum* type E were isolated at various developmental stages of growth and sporulation. Electron microscopy of thin sections showed that most of the granules were dispersed throughout the cytoplasm. Chemical analysis and electron microscopy showed that the granules were poly-β-hydroxybutyrate (PHB). The polymer began to accumulate after 8 h of growth, reaching 9 and 13% of the cell dry weight in the sporogenic and asporogenic strains, respectively, during early stationary phase. ^{14}C-acetate was readily incorporated into PHB. The rate of assimilation paralleled the production and utilization of PHB, indicating that the acetate served as its precursor. ^{14}C-butyric acid was not utilized to any significant extent. Most of the PHB which had accumulated in the sporogenic strain was catabolized during the development of the spore, but in the asporogenic mutant it remained essentially unchanged. The findings suggest that the PHB provides endogenous carbon and energy for the synthesis of spore-specific components required for spore maturation.

Cytoplasmic granulation has been observed in some cells of *Clostridium* spp. (8, 10, 13), but the granules have been considered to be carbohydrate in nature and have been given such names as polyside (10), amylopectin (13), and granulose (8). Poly-β-hydroxybutyrate (PHB) granules have not been detected in sporulating cells of clostridia so far as we know. Granules were not observed in the cells of *Clostridium botulinum* which had been assayed for PHB and were found to be negative by Day and Costilow (2).

In previous work with type E strains of *C. botulinum*, we observed that prior to the appearance of refractile spores, the cells had rapidly fermented glucose, accumulated acetate, and produced a densely granulated cytoplasm (unpublished data). In the present study, granules were isolated and identified as PHB, and their metabolism was followed during growth and spore formation.

MATERIALS AND METHODS

Microbial strains. Two nontoxigenic mutants (4) which were derived from *Clostridium botulinum* type E toxigenic ATCC 9564 were used in this study. The sporogenic mutant, MSp$^+$, yielded more than 80% spores after 48 h at 30 C and the asporogenic mutant,

RSpIIIa, was blocked at the forespore stage of sporulation (5).

Growth conditions and collection of samples. The active culture method in Trypticase-peptone-glucose broth (TPG) at 30 C was used for cell propagation (5). Samples for assays were withdrawn at intervals of 4 h for the 1st 24 h and again after 48 h.

Microscopy. Cultures were examined by phase-contrast microscopy and also stained by standard methods (12) for sudanophilic, iodophilic, and volutin granules. Electron microscopy techniques used for ultrastructural studies have been previously described (5). Carbon was used in place of palladium for shadow casting the granules (14).

Isolation and estimation of PHB. Cells were collected by centrifugation from 15-ml samples of culture and suspended in 9 ml of alkaline hypochlorite reagent (14). After 24 h at 37 C, lysis was confirmed by phase-contrast microscopy. The reaction mixture was centrifuged at 5,500 × *g* for 30 min, and the solid pellet was washed successively with water, acetone, and ether and dissolved in hot chloroform. After evaporation, the sample was treated with acetone-ether (2:1 vol/vol), and the precipitate was dissolved in 1 ml of hot chloroform. The sample was then precipitated with 2.5 ml of ether before drying for 12 h at 110 C. The white powder was dissolved in 0.5 ml of hot chloroform and heated for 10 min at 100 C after the addition of 10 ml of H_2SO_4. The solution was cooled and read at 235 nm against an H_2SO_4 blank (7). Purified extracts of PHB and sodium DL-β-

hydroxybutyric acid (Sigma) were used to prepare a calibration curve (E_{235} of 1.0 was given by 8.2 μg of PHB/ml). Duplicate samples were assayed twice, and the averages of four determinations were estimated.

Incorporation of acetate and butyrate. ^{14}C-acetic acid (U) (specific activity 16.5 mCi/mmol) or butyric acid-1-^{14}C (specific activity 57 mCi/mmol; Amersham/Searle Corp., Arlington Heights, Ill.) was added to the growth medium in amounts of 1 μCi/100 ml. Unlabeled sodium acetate or butyric acid was added to a final concentration of 2 $\times$ 10^{-3} M. The growth medium was inoculated with 10^6 cells of the MSp$^+$ or RSpoIIIa mutants and incubated at 30 C. PHB granules were isolated and purified as described above. The purified polymer was dissolved in hot chloroform and evaporated to 0.2 to 0.3 ml, and 10 ml of Brays scintillating fluid was added before counting for ^{14}C activity in a Packard scintillation counter.

RESULTS

Before the end of the log phase, granules were observed in the cytoplasm of sporogenic and asporogenic cells by phase-contrast microscopy. Stained cells showed that the granules were sudanophilic, whereas volutin granules were not observed, and the iodine-stained cells gave variable results. The granules were isolated, and chemical analysis before and after purification showed 70 and 96% PHB content, respectively, on the basis of total dry weight. The purity of the granules was confirmed by electron microscopy after shadow casting with carbon.

The biosynthesis of PHB occurred during growth of the sporogenic and asporogenic mutants of *C. botulinum* (Fig. 1). The polymer was first detected after 8 h of growth. The accumulation continued, reaching 9 and 13% dry weight of sporogenic and asporogenic cells, respectively, at the early stages of stationary phase. In
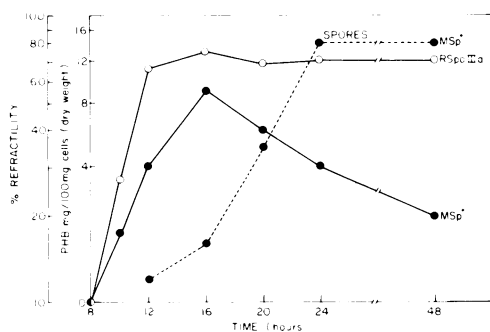
the sporogenic strain, the PHB content of the cells then decreased. At the free-spore stage, only about 2% dry weight was detected. In the asporogenic mutant, the amount of PHB which had accumulated in the aging cells remained essentially unchanged.

^{14}C-acetate of ^{14}C-butyric acid was incorporated into PHB of growing cells in "active culture" (Fig. 2). In the sporogenic and asporogenic mutants, acetate incorporation paralleled the accumulation and catabolism of the polymer. The specific activity of ^{14}C-acetate rapidly reached 1.2 $\times$ 10^3 counts per min per mg of PHB and did not change significantly. Butyric acid incorporation into PHB was low, with a specific activity of 0.2 $\times$ 10^3 counts per min per mg of PHB.

The electron micrographs of thin sections showed that granules in sporulating cells were dispersed throughout the cytoplasm, and a few were trapped within the forespore (Fig. 3 and 4). Purified granules which had been shadow casted with carbon are shown in Fig. 5.

DISCUSSION

The various developmental stages of *C. botulinum* examined for PHB included vegetative, forespore, endospore, and free spore (Fig. 6) as determined by phase-contrast and electron microscopy (unpublished data). The asporogenic mutant blocked at the forespore stages (5) accumulated many intracellular granules and
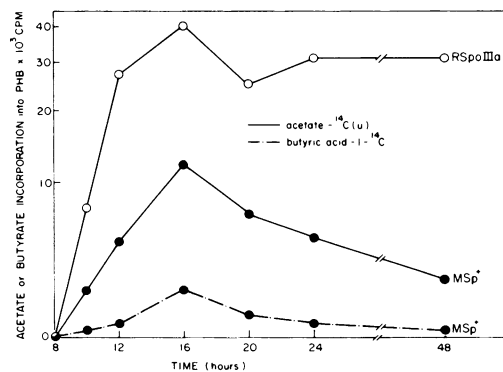
FIG. 1. *Poly-β-hydroxybutyrate (PHB) accumulation and degradation during the growth cycle of a sporogenic and an asporogenic mutant of C. botulinum. Cells were harvested at intervals, and granules were isolated, purified, and assayed for PHB spectrophotometrically. Refractility is expressed as percentage of refractile spores in total cells counted as determined by phase-contrast microscopy.*

FIG. 2. *Incorporation of ^{14}C-labeled acetate or butyrate into poly-β-hydroxybutyrate (PHB) during the growth cycle of a sporogenic and an asporogenic mutant of C. botulinum. PHB granules were isolated and purified from cells at intervals after growth in a medium containing 1 μCi of acetate-^{14}C or butyrate-1-^{14}C per 100 ml and assayed for ^{14}C radioactivity. The total counts/min at each interval were obtained by multiplying the specific activity (1.2 × 10^3 counts per min per mg of PHB) by total milligrams of PHB/100 mg of cell (dry weight).*
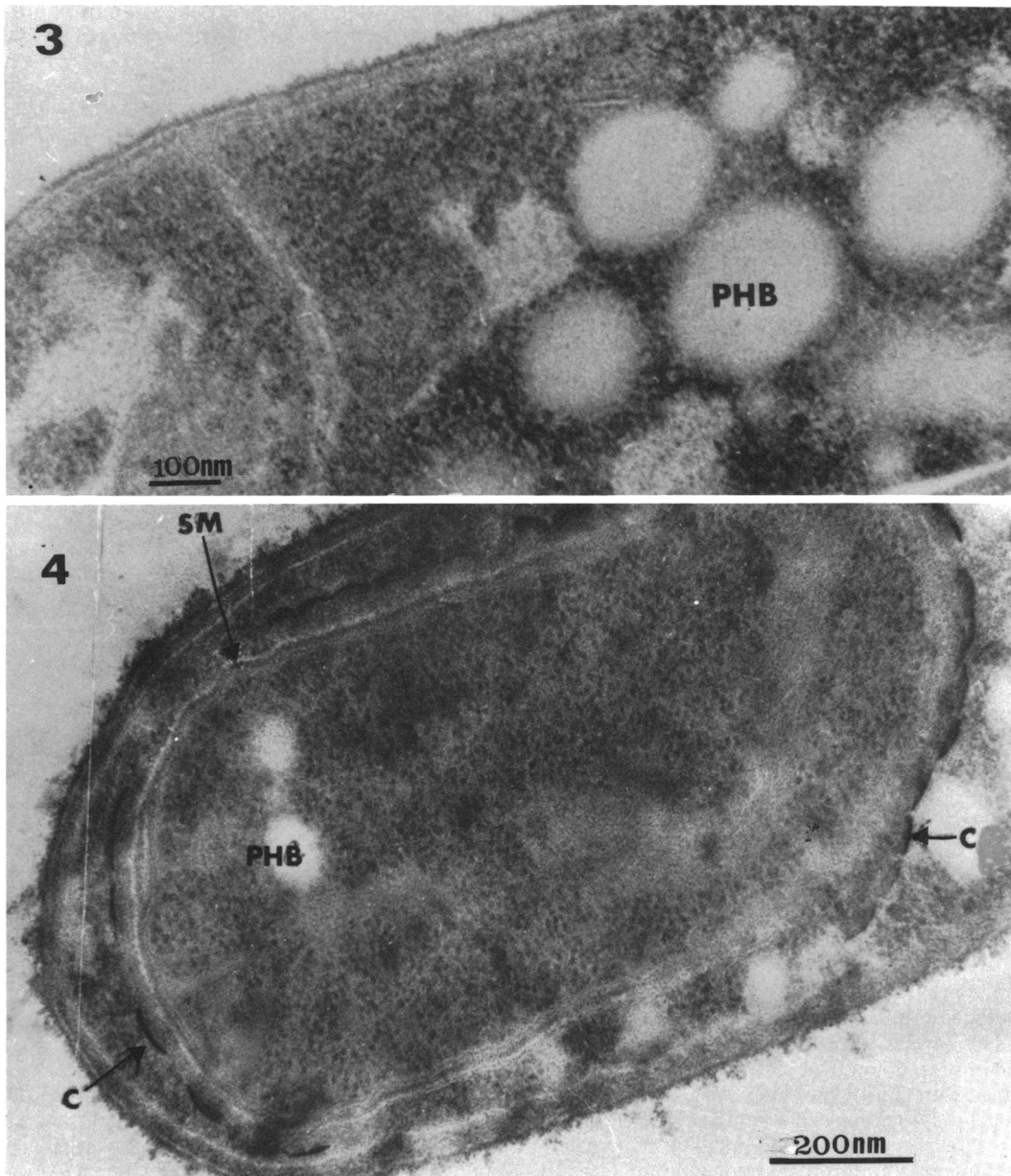
FIG. 3. *Longitudinal section of C. botulinum showing poly-β-hydroxybutyrate inclusions.*
FIG. 4. *Longitudinal section of forespore showing poly-β-hydroxybutyrate inclusions. SM refers to spore membrane; C to spore coat segments.*
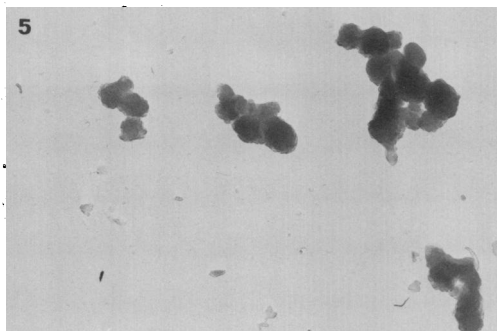
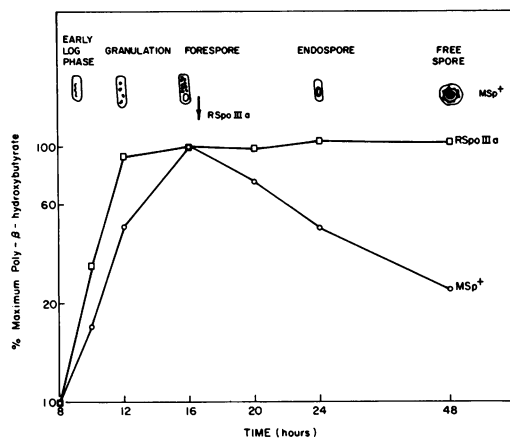FIG. 5. *Poly-β-hydroxybutyrate granules isolated from C. botulinum shadowed at 20° with carbon;* ×*12,800.*

FIG. 6. *Poly-β-hydroxybutyrate levels in sporogenic (O) and asporogenic cells (□), expressed in arbitrary units by using 100% as maximum accumulation. The sequential morphological stages of sporogenesis are indicated diagrammatically. The arrow (↓) indicates the developmental stage of blockage of the asporogenic mutant.*

hence provided a basis for comparison with the sporogenic mutant.

The results showed that the granules which accumulated by the start of the stationary phase were PHB, and they were seen throughout the cytoplasm with some in the matrix of the forespore (Fig. 3 and 4). The asporogenic mutant accumulated a larger amount of PHB, even in aging cells undergoing lysis (5). Because ^{14}C-acetate was readily incorporated into the PHB granules (Fig. 2) it would appear, as suggested for *Bacillus* spp. (3, 6, 9, 11), that one of the important roles of acetate was to supply carbon precursors via PHB for the synthesis of spore materials. As observed by Doudoroff and Stanier (3) when ^{14}C-butyric acid was used as

substrate, a low yield of PHB and reduced rate of breakdown resulted (Fig. 2), suggesting that butyric acid is an unlikely precursor.

The polymer was not utilized to any significant degree by the asporogenic mutant, suggesting an impaired mechanism for degradation (11). Furthermore, the failure of the asporogenic mutant to metabolize PHB could be due to a feedback or mass effect due to the developmental block at stage 3. On the other hand, most of the PHB was catabolized by the sporogenic strain. Only 2% PHB (dry weight) remained in the spores, which is in accord with the findings of Akashi (1) in spores of *Bacillus subtilis*. Because the polymer was catabolized between 16 and 48 h of the growth cycle, corresponding to stages 3 to 6 of sporulation (Fig. 6), it would appear that PHB granules are utilized as carbon and energy sources for spore maturation.

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