Over 90% of the complexed PHB is distributed throughout the cytoplasm, where it is covalently bonded with various proteins, especially those of ribosomes (3). There are conflicting reports about whether PHB is absent (11) or present within dormant bacterial spores. When reported to occur, the amounts are large (1, 13). Therefore, we undertook a reassessment of the nature and amount of PHB within the spores of four species, using rigorous cleaning procedures and sensitive, specific methods of analysis.

The dormant spores were cultivated and cleaned as described by Marquis et al. (6). Although seemingly clean by phase microscopic monitoring, the spores of *Bacillus cereus* T required further treatment with PHB depolymerase (isolated from *Alcaligenes faecalis*) to remove all exogenous PHB (12). The spores of *Bacillus megaterium* ATCC 19213, which lack exosporium, were decoated (6) so as to leave only their cortex-encased protoplast.

The usual methods for analysis of PHB involve first isolating PHB granules based on their stability to alkaline hypochlorite; insolubility in water, methanol and acetone; solubility in hot chloroform; and conversion to crotonic acid (CA) by concentrated sulfuric acid. The CA is then assayed by spectrophotometry (5) or chromatography (13). The method we used involves direct conversion of the PHB in intact spores to CA by treatment with hot concentrated sulfuric acid; extraction of the CA into dichloromethane; evaporation of the dichloromethane; separation of the CA by ion-exchange HPLC; identification by elution time, UV absorption and mass spectrometric comparison with standard CA; and quantification of the CA by measurement of the chromatographic elution peak area. The results were corrected for incomplete conversion of PHB to CA and for degradation of the CA over the time of the assay. The mean of three determinations was taken. The method measures the total PHB content, has a sensitivity of 10 ng, and has an accuracy of ±15% (3).

Protein–PHB complexes were analysed by a ‘Western’ immunoblotting technique. They were separated on a 10% polyacrylamide gel slab, transferred electrophoretically to a supported nitrocellulose sheet, probed with rabbit anti-PHB IgG that was marked with anti-rabbit IgG conjugated to alkaline phosphatase, developed with a synthetic substrate of the phosphatase, and stained with nitro blue tetrazolium (3). The complexes were compared with standard proteins of known molecular mass, which were analysed concurrently.

The PHB analyses indicated that the polymer does indeed occur within dormant bacterial spores, but only at the low concentrations characteristic of bacteria containing the complexed PHB rather than at the high concentrations characteristic of bacteria containing the granulated PHB. Mean PHB concentrations [mg (mg dry wt)⁻¹] of the spores were as follows: *Bacillus subtilis* biowar *niger*, 0-10; *B. megaterium* ATCC 19213, 0-38; *B. megaterium* ATCC 19213, decoated spores, 0-40; *Clostridium sporogenes* ATCC 7955, 0-44; *B. cereus* T, after treatment with PHB depolymerase, 0-80. The 10-fold lower concentrations of complexed PHB in spores than in vegetative cells may be attributed to the higher protoplast solids content and the additional integument layers (cortex, coat and exosporium) in spores. The retention of complexed PHB within the decoated spores of *B. megaterium* indicates localization of PHB within the protoplast, contradicting a report that PHB ‘may be
localized in the inner coat' (4). The complexed PHB in the spore protoplast presumably remains inactive until after germination and then functions as in vegetative cells.

Fig. 1 depicts the conjugation of complexed PHB with a broad array of proteins within dormant native spores of the four species. The significance of protein-PHB complexes is being studied in other bacterial cells (3).

The PHB concentrations we found are below the sensitivity and specificity limits of the Law & Slepecky analysis (5), thus explaining their inability to detect PHB in spores (11), and much below the 4-1 and 3-6 % dry weight reported to occur in B. cereus spores (1, 13). Although the latter authors took care to monitor and remove PHB granules originating from vegetative cells and contaminating the spore preparations, they apparently were not fully successful. B. cereus spores are notoriously contaminated with PHB granules, which apparently adhere to the exosporium. Only by treating these spores (even when seemingly clean by phase microscopic inspection) with PHB depolymerase were we able to rid them completely of exogenous PHB. This terminal enzyme treatment is recommended for general use in cleaning spores thoroughly.

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