Polyhydroxyalkanoate Inclusion Body-Associated Proteins and Coding Region in *Bacillus megaterium*

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Polyhydroxyalkanoic acids (PHA) are carbon and energy storage polymers that accumulate in inclusion bodies in many bacteria and archaea in response to environmental conditions. This work presents the results of a study of PHA inclusion body-associated proteins and an analysis of their coding region in *Bacillus megaterium* 11561. A 7.917-bp fragment of DNA was cloned and shown to carry a 4,104-bp cluster of 5 *pha* genes, *phaP*, *-Q*, *-R*, *-B*, and *-C*. The *phaP* and *-Q* genes were shown to be transcribed in one orientation, each from a separate promoter, while the remaining genes were transcribed divergently. Transfer of this gene cluster to *Escherichia coli* and to a PhaC mutant of *Pseudomonas putida* gave a Pha phenotype in both strains. Translational fusions to the green fluorescent protein localized PhaP and PhaC to the PHA inclusion bodies in living cells. The data presented are consistent with the hypothesis that the extremely hydrophobic protein PhaP is a storage protein and suggests that PHA inclusion bodies are not only a source of carbon, energy, and reducing equivalents but are also a source of amino acids.

Polyhydroxyalkanoic acids (PHA) are a class of aliphatic polyesters that accumulate in inclusion bodies in many bacteria and archaea in response to environmental conditions. Their physiological role in the cell is that of carbon and energy reserves and that of a sink for reducing power. The most-studied PHA have repeating subunits of \(-[O–CO(CH(R)(CH2))x-CO]–\), where the most common form is polyhydroxybutyrate, for which \(R = CH_3\) and \(x = 1\). The PHA biosynthetic pathway has been worked out for *Alcaligenes eutrophus* (17, 18, 44). In this organism two molecules of acetyl coenzyme A (acetyl CoA) are condensed by \(\beta\)-ketothiolase (PhaA), followed by a stereo-specific reduction catalyzed by NADPH-dependent reductase (PhaB) to produce the monomer \(D-(\rightarrow)\beta\)-hydroxybutyryl CoA, which is polymerized by PHA synthase (PhaC). These three *pha* genes are encoded on the *phaCAB* operon, which is constitutively expressed, but PHA is not constitutively synthesized. Alternative pathways for synthesis of the monomer in other organisms have been suggested, most notably in the *Pseudomonas* species where the side chain, \(R\), is longer than \(CH_3\) and its composition is influenced by carbon substrates in the growth medium (7, 45). In addition to being cloned from *A. eutrophus*, PhaC has been cloned from more than 20 different bacteria (26, 43). Other genes associated with PHA synthesis, *phaA*, *phaB*, *phaZ* (PHA depolymerase), and genes for inclusion body-associated proteins and other low-molecular-weight proteins of unknown function, have also been cloned from some of these bacteria, in many cases by virtue of the fact that they are clustered with *phaC*.

PHA inclusion bodies are 0.2 to 0.5 \(\mu\)m in diameter, but their structural details are largely unknown. They were described originally for some species of *Bacillus* (6, 8, 15, 30, 47) and later for many more bacteria, including *Pseudomonas, Alcaligenes,* and *Rhodococcus* species (5, 11, 12, 25, 42). Those from *Bacillus megaterium* were shown to contain 97.7% PHA, 1.87% protein, and 0.46% lipid, with protein and lipid forming an outer layer (15). More recent reports show the presence of a 14-kDa protein (GA14) on PHA inclusion bodies of *Rhodococcus ruber* (36, 37) and a 24-kDa protein (GA24) with similarities to GA14 on the inclusion bodies of *A. eutrophus* (48). These proteins are not essential for PHA accumulation but have been shown to influence the size of PHA inclusion bodies and the rate of PHA accumulation (37, 48). GA14 and GA24 have been named phasins due to some similarities with oleosins, which are proteins on the surface of oil bodies in plant seeds (21). Proteins with similarity to GA24 are widespread in PHA-accumulating bacteria (49). We have previously described the pattern of PHA inclusion body growth and proliferation throughout the growth cycle of *B. megaterium* (32). We now present the results of a study of PHA inclusion body-associated proteins from *B. megaterium* and the cloning and analysis of their coding region. The transcription starts were identified, the functional expression of some of the genes was confirmed in *Escherichia coli* and in a PHA-negative mutant of *Pseudomonas putida*, and PhaP and PhaC were localized to PHA inclusion bodies throughout growth.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1.

**Media and growth conditions.** Cultures were grown at 37°C (unless otherwise stated) in liquid media, aerated by rotation at 250 rpm in either Luria-Bertani broth (LB) (33) or M9 minimal salts (Life Technologies) with 1% (wt/vol) glucose. For growth on plates, the above media with 1.5% agar (Sigma catalog no. A4550) were used. For plasmid selections, the appropriate antibiotics were included in the media: ampicillin (200 \(\mu\)g/ml [AMP200]), chloramphenicol (25 \(\mu\)g/ml [CM25]), erythromycin (200 \(\mu\)g/ml [EM200]), or tetracycline (12.5 \(\mu\)g/ml [TC125]) for plasmid selection in *E. coli*; CM25 alone or EM25 plus lincomycin (25 \(\mu\)g/ml [LM25]) for plasmid selection in *B. megaterium*; and CM25 or TC25 for selection in *Pseudomonas*.

**Separation of polypeptides associated with PHA inclusion bodies.** Inclusion bodies were purified as previously described (32) followed by suspension in TE (10 mM Tris-HCl [pH 8], 1 mM EDTA) with 2% sodium dodecyl sulfate (SDS). An equal volume of 2× sample buffer was added prior to boiling for 5 min, and samples were centrifuged for 3 min to pellet PHA; the supernatant was loaded on an SDS–12% polyacrylamide gel and run at 8 mA overnight at 4°C to separate the proteins. The gel was stained with Coomassie blue for 5 min prior to transfer of proteins to a polyvinylidene difluoride (PVDF) membrane by using a semidy electroblotter at 400 mA for 45 min. The membrane carrying the proteins...
interest was cut for use in N-terminal amino acid sequence determination by Edman degradation using a minimum of 200 pmol of each protein.

Transformations. E. coli and P. putida were transformed by electroporation of competent cells using an electroporator (Eppendorf) and following the manufacturer’s instructions. P. putida was transformed by a biolistic transformation procedure (39).

Cloning the pha region. Purification of genomic and plasmid DNA, Southern blotting, hybridization, and cloning were performed by standard procedures (38).

To clone the DNA sequences that coded for the two most abundant proteins on the phaB, phaC region.

The transcription start points were mapped in the B. megaterium genomic DNA, were purified from agarose following polymerase chain reaction (PCR) amplification using primers designed to be amplified in the range of 1-2 kb.

TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5α</td>
<td>deoR endA1 gyrA96 hsdR17 (r~ m~) recA1 relA1 supE44 thi-1 ΔlacZYA-argF169 Δ80lacZΔM15 F- λ-</td>
<td>Clontech</td>
</tr>
<tr>
<td>B. megaterium 11561</td>
<td>Wild type; used to clone pha genes</td>
<td>ATCC</td>
</tr>
<tr>
<td>P. oleovorans 29347</td>
<td>PHA-positive control</td>
<td>ATCC</td>
</tr>
<tr>
<td>P. putida GpPl104</td>
<td>PHA-negative mutant; phaC deletion mutant</td>
<td>22</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescriptIIISK</td>
<td>Cloning vector; ColE1 oriV Ampγ</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pGFPuv</td>
<td>Source of gfp gene; ColE1 oriV Ampγ</td>
<td>Clontech</td>
</tr>
<tr>
<td>pHPS9</td>
<td>Bacillus-E. coli shuttle vector; ColE1 and pTA1060 oriV Emγ Cmγ</td>
<td>16</td>
</tr>
<tr>
<td>pSUP104</td>
<td>Pseudomonas-E. coli shuttle vector; Q-type and mini15 oriV Emγ Teγ</td>
<td>40</td>
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<td>pGM1</td>
<td>EcoRI in phaB to HindIII in phaC, cloned into the EcoRI-HindIII sites of pBluescriptIIISK; Ampγ</td>
<td>This study</td>
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<tr>
<td>pGM6</td>
<td>Pfl in phaB to EcoRI in ykM, cloned into the Pfl-I-EcoRI sites of pBluescriptIIISK; Ampγ</td>
<td>This study</td>
</tr>
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<td>pG7</td>
<td>EcoRI in phaB to EcoRI in ykM, cloned into the EcoRI site of pHPS9; Cmγ</td>
<td>This study</td>
</tr>
<tr>
<td>pG7H</td>
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<td>This study</td>
</tr>
<tr>
<td>pG7I</td>
<td>HindIII upstream of ykO to EcoRI in ykM, cloned into the HindIII-EcoRI sites of pBluescriptIIISK; Ampγ</td>
<td>This study</td>
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<tr>
<td>pG7G</td>
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<td>This study</td>
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<tr>
<td>pC/GFP2</td>
<td>PhaC::GFP out-of-frame fusion plasmid; fragment shown in Fig. 4A cloned in pBluescriptIIISK; Ampγ</td>
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<td>pC/GFP3</td>
<td>PhaB::GFP in-frame fusion plasmid; fragment shown in Fig. 4B cloned in pBluescriptIIISK; Ampγ</td>
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<tr>
<td>pG7M13</td>
<td>PhaB::GFP in-frame fusion plasmid; fragment shown in Fig. 4C cloned in pHPS9; Emγ Lmγ</td>
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<td>pG7M13C</td>
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<td>pG7M16.2</td>
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<td>pG7M107</td>
<td>EcoRI in phaB to EcoRI in ykM, cloned as a BamHI-SalI fragment from pGM7, into the BamHI and SalI sites of pSUP104; Cmγ</td>
<td>This study</td>
</tr>
<tr>
<td>pDR1</td>
<td>Pfl in phaB to EcoRI in ykM, cloned as a Smal-EcoRV fragment from pGM6 into the two DruI sites of pSUP104 in same orientation as the Cm gene, with phaC expressed from the Cm promoter; Teγ</td>
<td>This study</td>
</tr>
</tbody>
</table>

### Strains

Strains  
- **E. coli DH5α**  
- **B. megaterium 11561**  
- **P. oleovorans 29347**  
- **P. putida GpPl104**

### Plasmids

- **pBluescriptIIISK**  
- **pGFPuv**  
- **pHPS9**  
- **pSUP104**  
- **pGM1**  
- **pGM6**  
- **pG7**  
- **pG7H**  
- **pGM7**  
- **pG7M13**  
- **pG7M13C**  
- **pG7PF3**  
- **pG7M16.2**  
- **pG7M107**  
- **pDR1**

### Relevant Characteristics

- **Cloning vector**
- **Source of gfp gene**
- **Bacillus-E. coli shuttle vector**
- **Q-type and mini15 oriV**
- **EcoRI in phaB to HindIII in phaC**
- **EcoRI in phaB to EcoRI in ykM**
- **HindIII upstream of ykO to Pfl in phaB**
- **HindIII upstream of ykO to EcoRI in ykM**
- **PhaC::GFP out-of-frame fusion plasmid**
- **PhaB::GFP in-frame fusion plasmid**
- **GFP localization control plasmid**
- **PhaP::GFP in-frame fusion plasmid**
- **GFP localization control plasmid**
- **GFP in-frame fusion plasmid**
- **EcoRI in phaB to EcoRI in ykM, cloned as a BamHI-SalI fragment**
- **Pfl in phaB to EcoRI in ykM, cloned as a Smal-EcoRV fragment**

### Source or Reference

- **Stratagene**
- **Clontech**
- **ATCC**
- **American Type Culture Collection**
- **Origin of replication**
B. megaterium was cloned, sequenced, and characterized. It was shown to carry eight complete ORFs and one incomplete ORF (Fig. 2; Table 2). Genes in this region were assigned on the basis of homology to known sequences, N-terminal amino acid sequences, putative ribosome binding sites, and operon location. The complement and arrangement of genes flanking the pha genes in B. megaterium are very similar to those in a region of Bacillus subtilis 168 (Fig. 2). This strain is negative for PHA, and no known pha genes or sequences occur in its genome, for which the complete sequence is available (24). In place of pha genes in this region of B. subtilis are ykrK, ykrL, and ykrK, respectively, code for putative proteins similar to two unknown proteins and a probable heat shock protein.

**pha promoters.** The transcription start nucleotides in the pha region were determined. Primer extension products run on 8% denaturing polyacrylamide gels showed a single band from each reaction mixture, indicating one transcript, while control reaction mixtures in which RNA was omitted showed no bands. The extension products run alongside sequencing reaction products obtained with the same primer (Fig. 2C) identified the 5' ends of the transcripts, thus allowing the putative promoter sequences at approximately −10 and −35 bp for phaP, Q, and -R to be identified. The arrangement of genes in the pha cluster of B. megaterium is unique among those already published, and phaA is notably absent. The phaF, Q, -R, and -C genes were shown to be in a 4,104-bp region, with phaP and -Q transcribed in one orientation, each from a separate promoter, whereas phaR, -B, and -C were divergently transcribed from a promoter in front of phaR. The putative promoters responsible for transcription of phaQ and phaR, -B, and -C show strong similarity to both B. subtilis sigma A type (34) and E. coli sigma 70 type (14) promoters, which can express constitutively. This is in keeping with previous data for A. eutrophus showing that phaC is constitutively synthesized, but PHA is not constitutively accumulated (19). The third putative promoter in this region, the phaP promoter, resembles a sigma D-type promoter known to control the expression of a regulon of genes associated with flagellar assembly, chemotaxis, and motility (13, 20, 46). In B. subtilis sigma D is expressed in the exponential phase and peaks in late exponential phase of growth. This parallels the pattern of PHA accumulation previously described for B. megaterium 11561 (32). However, further experiments are required to test the hypothesis that PHA accumulation is regulated by sigma D or products of its resulting transcripts. The phaP gene has 18-bp duplicate sequences that could base pair to form a rho-independent terminator close to its translational stop codon (Fig. 2B). The fact that the −35 promoter region of sspD is within this putative hairpin structure suggests that transcription of phaP and sspD could be mutually exclusive, thus allowing the expression of phaP to play a regulatory role in the expression of sspD (which codes for spore specific storage protein).

**pha genes and their products.** The deduced amino acid sequence of PhaP shows a 20-kDa extremely hydrophilic protein with no obvious similarity to known sequences. Inclusion body-associated low-molecular-weight proteins (phasins) have been described in many bacteria (49), but for those for which sequences were available we found no similarities of identifiable significance with PhaP of B. megaterium. PhaP does not have an obvious membrane anchoring domain, nor can it be described as an oleosin-like protein as was described for that of R. ruber (36). Low-molecular-weight, PHA inclusion body-abundant proteins obviously play an important role in PHA-producing cells, since they are involved in determining inclusion body size and shape and are present in quantities up to 5% of total protein in the case of PHA-producing A. eutrophus (48). It is interesting that the amino acid sequences of phasin

**FIG. 1. PHA inclusion body-associated proteins.** Shown are the results of SDS-polyacrylamide gel electrophoresis of proteins released from purified PHA inclusion bodies. The bands were visualized by staining with Coomassie blue. Lane 1, molecular mass markers (14, 18, 29, 43, 68, and 97 kDa); lane 2, proteins from inclusion bodies of cells harvested in late exponential growth phase; lane 3, same as lane 2 except this part of the gel was stained following 45 min transfer of proteins (seen in lane 2) to PVDF membrane.
proteins are so dissimilar, even in closely related bacteria. Some similarity between such proteins would be expected in closely related bacteria, were they to have a role in inclusion body biogenesis; however, conservation of sequence would be entirely unnecessary should they have a role as storage proteins.

The deduced amino acid sequences of PhaQ and PhaR also revealed small hydrophilic proteins with no significant identifiable similarity to known proteins. Figure 1 (lane 2) shows that purified inclusion bodies have proteins represented by bands of the approximate sizes of PhaQ (17 kDa) and -R (23 kDa), but the roles of these proteins are unknown. They may be non-orthologous replacements for the small putative gene products, whose roles are also unknown, encoded in known pha gene clusters. The deduced amino acid sequence of PhaB is very similar in size and amino acid sequence to known phaB and fabG gene products (Table 2). The deduced amino acid sequence of PhaC shows that while it has low homology overall to known PhaC proteins, it is most similar to that of Thiocystis violacea, Synechocystis sp., and C. vinosum. PhaC proteins from these three bacterial strains, respectively, have 355, 378, and 355 amino acids, while PhaC from B. megaterium has 362 amino acids. All other PhaC proteins studied are larger in size and range from 559 amino acids for that of Pseudomonas oleovorans (22) to 636 amino acids for that of Rhizobium etli (3). Alignment studies of sequences of all known PhaC proteins show that each of the four small PhaC proteins is missing...
approximately 150 amino acids from the N terminus and 50 amino acids from the C terminus; this is demonstrated for PhaC from B. megaterium and P. oleovorans in Fig. 3.

**Expression of B. megaterium pha genes in E. coli and P. putida.** Functionality of the B. megaterium putative pha gene cluster was tested in E. coli, which is naturally PHA negative, and P. putida GPp104, a PhaC− mutant. Plasmids carrying one or more of these genes were introduced, and the resulting transformants were tested for PHA accumulation following growth on LB or M9 medium with various carbon sources and the appropriate antibiotic for plasmid selection. E. coli carrying pGM7 or pGM10 accumulated low levels of PHA while E. coli carrying pGM1 or pGM6 accumulated no PHA. Fluorescence microscopy of Nile blue A-stained cells showed that ~1 cell in 20 had one or a few inclusion bodies and the quantity of PHA produced was ~5% of cell dry weight. Since E. coli does not have PhaA, a low level of PHA or no PHA is the expected result. However, in *Pseudomonas*, in which PhaA is not known to be required, *P. putida* GPp104(pGM107) accumulated PHA on rich as well as minimal medium with various carbon sources to >50% of cell dry weight, and 90 to 100% of cells appeared full of PHA (Table 3). The positive control P. oleovorans, (equivalent to wild-type *P. putida*) accumulated PHA only when grown on longer-chain carbon sources, and not on LB. No PHA was accumulated by the negative control or by *P. putida* carrying PhaC alone (pDR1). These results showed that this *B. megaterium* gene cluster is functional in both *E. coli* and *P. putida*. PhaC alone was insufficient to complement PhaC− *P. putida* or to synthesize PHA in *E. coli*. Furthermore, since *Pseudomonas* cannot synthesize PHA when grown in LB, these data indicate that PhaB can function to supply substrate to PhaC in *P. putida*. However, these data do not exclude the possibility that PhaP, -Q, or -R is necessary for PHA accumulation.

**Localization of PhaP and PhaC.** Proteins associated with purified PHA inclusion bodies may not accurately reflect the localization of these proteins within the growing cell. Visualization of pha::gfp gene product fusion proteins in living cells throughout culture growth is a useful method for determining both the localization of the pha gene products and their comparative levels in growing cells. PhaP and PhaC, as fusion proteins (Fig. 4), localized to PHA inclusion bodies at all time points tested throughout growth of *B. megaterium* 11561. The negative control (pHPS9) showed no fluorescence at any time point. The localization control (pGM13C) showed nonlocalized green fluorescence at all time points. The profiles of PHA accumulation in these two control strains were similar to that of the wild-type, where the quantity of PHA decreased during the lag phase, increased during the exponential phase, and

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of amino acids</th>
<th>Mol mass (Da)</th>
<th>Isoelectric point</th>
<th>Homologous known or putative gene (accession no.)</th>
<th>Identity (%)</th>
<th>Similarity (%)</th>
<th>Function or putative function (reference[s])</th>
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<tbody>
<tr>
<td>ykoY</td>
<td>271</td>
<td>29,996</td>
<td>6.89</td>
<td>YkoY of <em>B. subtilis</em> (Z99110)</td>
<td>64</td>
<td>73</td>
<td>Toxic anion resistance protein (24)</td>
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<td>ykoZ</td>
<td>236</td>
<td>27,666</td>
<td>9.36</td>
<td>YkoZ of <em>B. subtilis</em> (Z99111)</td>
<td>57</td>
<td>74</td>
<td>RNA polymerase sigma factor (24)</td>
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<tr>
<td>xspD</td>
<td>65</td>
<td>7,027</td>
<td>8.58</td>
<td>SspD of <em>B. megaterium</em> (P10572)</td>
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<td>87</td>
<td>Spore-specific, DNA binding protein (10)</td>
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<td>7.39</td>
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<td>ND</td>
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<td>55</td>
<td>71</td>
<td>Na+ transporting ATP synthase (24)</td>
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*Accession numbers are from SWISS-PROT, EMBL, or DDBJ databases.
* Partial protein.
* ND, not determined.

FIG. 3. Pairwise alignment of PhaC from *B. megaterium* (B. meg.) (this study) and *P. oleovorans* (P.ole.) (SWISS-PROT accession no. P26494); amino acid identities are boxed in black. The Clustal method with a PAM250 residue weight table was used.
PhaC, monitored as a PhaC::GFP fusion protein in pGM13 (data not shown), showed a profile of expression similar to that of PhaP with one exception: PhaC did not reduce in level (data not shown), showed a profile of expression similar to that of cells carrying PhaP::GFP, except that the increased quantity of PhaC in the early to mid-exponential phase, decreased in mid- to late exponential phase, and increased during stationary phase growth. The rapid decrease of PhaP in lag phase is consistent with PhaP being a storage protein that is degraded as a source of amino acids. The profile of PHA accumulation in these cells (carrying pGM16.2) followed a pattern similar to that of PhaP except that PHA decreased only in the lag phase and continued to accumulate throughout other phases of culture growth. This data is consistent with PHA inclusion bodies’ being a source of carbon, reducing equivalents and amino acids when the organism is first provided with fresh medium. Possible explanations as to why the level of PhaP and not PHA decreased at mid- to late exponential phase are that either PhaP was synthesized at a lower rate than PHA was or PhaP was used as a source of amino acids at this phase of growth, or both scenarios may apply.

PhaC, monitored as a PhaC::GFP fusion protein in pGM13 (data not shown), showed a profile of expression similar to that of PhaP with one exception: PhaC did not reduce in level during lag phase growth. It did, however, reduce in level in mid- to late exponential phase growth, as did PhaP. The profile of PHA accumulation in these cells carrying PhaC::GFP was similar to that of cells carrying PhaP::GFP, except that the PHA level did not reduce during lag phase growth. It is reasonable to assume that the increased quantity of PhaC in the cell is a likely explanation since PhaC remained functional in the fusion protein PhaC::GFP. This was indicated by the fact that E. coli DH5a(pC/GFP3) and E. coli DH5a(pGM7) accumulated PHA to equivalent low levels, while the host strain alone, or carrying pGFPuv, accumulated no PHA, as visualized by fluorescence microscopy of Nile blue A-stained cells. The reduction in level of PhaC in mid- to late exponential phase, as was also seen with PhaP, is consistent with both PhaC’s and PhaP’s being synthesized at a lower rate than PHA is.

In cells of all growth phases, inclusion bodies were rarely visible under light in stained heat-fixed cells while larger inclusion bodies were visible by phase-contrast microscopy of living cells (Fig. 5C to F). In older cultures (2 days and older) some cells were lysed and showed PhaP::GFP and PhaC::GFP localized to free PHA inclusion bodies (Fig. 5D). Both free and intracellular inclusion bodies had doughnut-shaped localization of GFP at some focal planes while at other focal planes the same inclusion bodies appeared completely covered in GFP. We interpret this data as a difference in quantity of GFP that is visible when viewed through the edge or the center of the inclusion bodies.

Concluding remarks. This is the first report of PHA inclusion body-associated proteins and their coding region in B. megaterium. The phaP, -Q, -R, -B, and -C genes can be positioned in the B region of the B. megaterium map due to linkage to the sspD gene (46), provided that strains 11561 and QM B1551 have similar genetic maps. PhaP, -Q, and R are extremely hydrophilic proteins, with no discernible sequence similarities to known proteins. PhaP localized to PHA inclusion bodies in living cells. In addition to a possible role in inclusion body biogenesis, the data are consistent with PhaP’s being a storage protein. PhaB and -C have homology to known PHA proteins. The sequence of PhaB is more like that of FabG than like other PhaB proteins, and our data suggest that it can function to provide substrate to the B. megaterium PhaC in a PhaC mutant of P. putida, thus allowing PHA accumulation. PhaC is among the smallest of the known PhaC proteins and localizes to PHA inclusion bodies in living cells. The significance of PhaC and PhaP localization in the mechanism of PHA inclusion body biogenesis and PHA accumulation is being further investigated. The analysis of the pha locus of B. megate-

### Table 3. Cells with PHA as a percent* of total cells following growth on different carbon sources

<table>
<thead>
<tr>
<th>Substrate(s) (no. of C atoms)</th>
<th>B. megaterium (pGM7)</th>
<th>P. oleovoransb</th>
<th>P. putida GPp104(pSUP104)c</th>
<th>P. putida GPp104(pGM107)d</th>
<th>P. putida GPp104(pDR1)e</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>LB-1% glucose</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>92</td>
<td>0</td>
</tr>
<tr>
<td>M9-12 mM caproate (6)</td>
<td>No growth</td>
<td>88</td>
<td>0</td>
<td>100f</td>
<td>0</td>
</tr>
<tr>
<td>M9-12 mM octanoate (8)</td>
<td>No growth</td>
<td>90</td>
<td>0</td>
<td>92</td>
<td>0</td>
</tr>
</tbody>
</table>

*a 100%, PHA in all cells; 0%, no PHA in any cell; data averaged from more than five fields of each of three different cultures; error <5%.

*b Positive control.

*c Negative control, vector only.

*d phaPQRBC (r indicates presence of N terminus only).

*e phaC.

f Cell shape distorted by large quantity of PHA.
rium not only provides further comparative data on pha genes and their products but also allows a comparison of this locus with its counterpart in B. subtilis.

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