Polyhydroxyalkanoic acids (PHAs) are a family of bacterial polyesters synthesized by a wide range of organisms under conditions of nutrient limitation in the presence of an excess carbon and energy source (1). These polymers have attracted significant attention for their potential commercial exploitation as biodegradable plastics (22). The most abundant and best-studied PHA is poly-β-hydroxybutyrate (PHB), a linear, unbranched homopolymer built up of (R)-3-hydroxybutyric acid units.

The physiology and molecular genetics of PHB biosynthesis have been extensively studied in *Alcaligenes eutrophus* (33). More recently, genes involved in PHA metabolism have also been cloned from numerous other organisms (see the review in reference 32). In most organisms, PHB is synthesized via a three-step pathway which involves the condensation of two molecules of acetyl coenzyme A (acetyl-CoA) to acetoacetyl-CoA via a β-ketothiolase (PhaA), reduction of acetoacetyl-CoA to d-(-)-β-hydroxybutyryl-CoA via an NADPH-dependent acetoacetyl-CoA reductase (PhaB), and polymerization to PHB via a PHA synthase (PhaC) (33).

Recently, the PHA synthase gene was cloned from an *Acinetobacter* strain isolated from a modified activated sludge treatment plant (31). *Acinetobacter* species have been suggested to be the major organisms responsible for enhanced biological phosphate removal from wastewater treated in alternating anaerobic/aerobic activated sludge systems (5). Biochemical models of this overall process involve the breakdown of polyphosphate and synthesis of PHB during the anaerobic stage and breakdown of PHB and production of polyphosphate in the subsequent aerobic stage (8). Strains of *Acinetobacter* which can accumulate up to 10% phosphorus (10) and 15% PHB (28) on a dry weight basis have been isolated.

In *A. eutrophus*, all three PHA biosynthetic enzymes are synthesized constitutively. Regulation is achieved at the enzymatic level as a result of inhibition of the β-ketothiolase by free acetyl CoA (33). The evidence presented in this report suggests that the alternative mechanism for the regulation of PHB biosynthesis in *Acinetobacter* spp., which involves activation of the PHB biosynthetic genes under conditions of phosphate starvation, although the phosphate uptake systems in *Acinetobacter* spp. resemble the two major transport systems of *Escherichia coli* (35), little is known of these processes at the molecular level. In *E. coli*, a number of genes in the phosphate (pho) regulon which are involved in the transport and use of phosphate are expressed coordinately when the cell is starved of phosphate (36). The expression of these genes is positively regulated by the product of the *phoB* gene (PhoB), which activates transcription by binding to an 18-bp consensus pho box sequence found 10 bp upstream of the −10 region of promoters in the pho regulon (21, 37).

In this study, we have sequenced the genes involved in PHB biosynthesis from an *Acinetobacter* strain isolated from activated sludge. Two sequences displaying similarity to *E. coli* pho box consensus sequences were identified within the *Acinetobacter* PHA biosynthetic gene locus. We demonstrate that increased transcription of these genes under conditions of phosphate starvation in *Acinetobacter* or *E. coli* transforms is associated with one of these regulatory regions.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Acinetobacter* strain RA3849 was isolated from a modified activated sludge treatment plant and identified by a transformation test (15). This strain was grown at 30°C on a defined medium containing acetate as a sole source of carbon and energy (referred to as ADM) (2). For PHB accumulation, the concentration of NaH₂PO₄·2H₂O in ADM was reduced to 0.001% (wt/vol), and this medium is referred to as ADM (limiting P). *E. coli* was grown at 37°C in either Luria-Bertani (LB) medium (29) or Tris-glucose (TG) medium with either low or high phosphate concentrations (11). Ampicillin was added to LB broth at 50 μg/ml and to TG medium at 2 mg/ml as required.

**Recombinant DNA techniques.** All DNA manipulations were performed by standard procedures (29). Oligonucleotide primers were synthesized on an Applied Biosystems 381A oligonucleotide synthesizer and eluted from columns as instructed by the manufacturer. DNA sequencing was performed with the PRISM Ready Reaction DyDeoxy Terminator Cycle Sequencing Kit (Applied
TABLE 1. Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acinetobacter</strong></td>
<td><strong>PHB positive</strong></td>
<td>31</td>
</tr>
<tr>
<td>RA3849 E. coli</td>
<td>thi</td>
<td>3</td>
</tr>
<tr>
<td>CA8000</td>
<td>thi phoB62</td>
<td>17</td>
</tr>
<tr>
<td>pJKD1226</td>
<td>pJKD1536</td>
<td>This study</td>
</tr>
<tr>
<td>pUC19</td>
<td>pJKD1341</td>
<td>This study</td>
</tr>
<tr>
<td>primers1784 and 2013 were used</td>
<td>pJKD1425</td>
<td>This study</td>
</tr>
<tr>
<td>201-bp PCR product from</td>
<td>pJKD1537</td>
<td>This study</td>
</tr>
<tr>
<td>primers 1784 and 2013 containing pho-activated</td>
<td>pJKD1539</td>
<td>This study</td>
</tr>
<tr>
<td>promoter from phaBAc upstream region in pKK232-8</td>
<td>pJKD1646</td>
<td>This study</td>
</tr>
</tbody>
</table>

To clone this upstream promoter region, a 4.4-kb EcoRI overlapping fragment was identified by Southern hybridization and ligated into pUC19 to form pJKD1341 (Fig. 1A). Sequence comparison of this clone with that of the corresponding region of pJKD1226 confirmed the direct overlap of these two fragments in the *Acinetobacter* strain RA3849 genome. A 5.6-kb HindIII-XbaI fragment containing the three PHA biosynthetic genes and the upstream promoter region was then obtained from these clones and ligated into pUC19 to form pJKD1425 (Fig. 1A). Unlike pJKD1226, this plasmid did promote PHB accumulation in its *E. coli* host under appropriate conditions, as determined by staining with the lipophilic stain Sudan black (data not shown).

**Sequence analysis of the PHA biosynthetic gene locus.** The complete nucleotide sequence of the 5.6-kb HindIII-XbaI fragment in pJKD1425 was obtained by sequencing on both strands, using numerous subclones and several synthetic oligonucleotides. Four adjacent open reading frames with appropriately positioned ribosome binding sites were identified. These open reading frames were referred to as *phaB*Ac, ORF1, *phaC*Ac, and *phaA*Ac, respectively. The overall molar G+C content of this region was 42%, a value consistent with the G+C content for the total genomic DNA of *Acinetobacter* spp. (38 to 45 mol% G+C) (14). No potential transcriptional terminators were identified within this locus.

The primary structure of the deduced *phaB*Ac gene product revealed strong similarity to the acetocetyl-CoA reductases of *A. eutrophus* (47.2% identical amino acids), *Chromatium vinosum* (45.9%), and *Zoogloea ramigera* (47.3%) (Fig. 2). Similarly, the deduced amino acid sequence from the nucleotide sequence of the *phaA*Ac gene showed significant similarity to the primary structures of β-ketothiolases from *A. eutrophus* (63.1% identical amino acids), *C. vinosum* (62.2%), *Z. ramigera* (53.7%), *E. coli* (42%), and *Saccharomyces uvarum* (41.5%) (Fig. 3). The *Acinetobacter phaC*Ac gene product has previously been characterized and shows significant similarity to other PHA synthase proteins (31).

A fourth open reading frame (ORF1) was also identified within the *Acinetobacter* strain RA3849 PHA biosynthetic gene locus. ORF1 could potentially encode a 13-kDa protein. The primary structure of the putative ORF1 gene product did not display significant similarity to any other protein sequences in the database. If ORF1 is expressed, its gene product may possess a function similar to that of small proteins identified at
the surface of PHA granules in other bacterial species (12, 13, 24).

Northern blot analysis. To study the regulation of PHA biosynthesis in *Acinetobacter* spp. and to determine the lengths of mRNA transcripts, Northern hybridizations were performed. Because of mRNA degradation, exact transcript sizes could not be ascertained. However, a dramatic increase in hybridizing material was observed in *Acinetobacter* strain RA3849 grown in ADM (limiting P) versus ADM (data not shown). These preliminary results suggested that PHB synthesis in *Acinetobacter* spp. is regulated at the transcriptional level.

Identification of transcriptional start sites and putative promoters. Primer extensions were performed as an alternative method of analyzing transcription of the *Acinetobacter* PHA biosynthetic genes. Oligonucleotides were designed in the 5' untranslated regions (UTRs) of the genes coding for PHA synthases and PHA degradases.

FIG. 1. (A) Physical map of the *Acinetobacter* strain RA3849 PHA biosynthetic gene locus reconstructed from two overlapping clones. The left-hand Sau3AI site of pJKD1226 maps at nucleotide 989 in Fig. 2, which lies within the putative *pho* box consensus sequence identified upstream of the *phaB* coding region. (B) Regions of the *Acinetobacter* strain RA3849 PHA biosynthetic gene locus cloned into the promoter selection vector pK232 and corresponding CAT signals obtained when plasmids were introduced into *E. coli* wild-type and *phaB* mutant strains and grown in the presence of limiting-phosphate (Li Pi) or excess-phosphate (Ex Pi) concentrations. CAT units are expressed as nanograms of CAT per microgram of protein. β-Lactamase activity was measured to correct for plasmid copy number effects and expressed as picomoles per minute per microgram of protein. Results are the averages of at least two independent experiments. Abbreviations: E, EcoRI; H, HindIII; HI, HindII; S, SphI; Sa, Sau3AI; X, XbaI.

VOL. 177, 1995 TRANSCRIPTIONAL REGULATION OF pha GENES BY PHOSPHATE 4503 on July 8, 2017 by guest http://jb.asm.org/ Downloaded from

FIG. 2. Alignment of the amino acid sequence deduced from the nucleotide sequence of the *Acinetobacter* (Ac) gene *phaB* (this study) with sequences of the acetoacetyl-CoA reductases of *A. eutrophus* (Ac) (26), *C. vinosum* (Cv) (18), and *Z. ramigera* (Zr) (27). Residues identical to those of the *Acinetobacter* acetoacetyl-CoA reductase are indicated by dots; gaps introduced into the alignment are indicated by dashed lines.

FIG. 3. Alignment of the amino acid sequence deduced from the nucleotide sequence of the *Acinetobacter* (Ac) gene *phaA* (this study) with sequences of the β-ketothiolases of *A. eutrophus* (Ac) (26), *C. vinosum* (Cv) (18), and *Z. ramigera* (Zr) (27). Residues identical to those of the *Acinetobacter* β-ketothiolase are indicated by dots; gaps introduced into the alignment are indicated by dashed lines.

FIG. 4. Alignment of the amino acid sequence deduced from the nucleotide sequence of the *Acinetobacter* (Ac) gene *phaA* (this study) with sequences of the β-ketothiolases of *A. eutrophus* (Ac) (26), *C. vinosum* (Cv) (18), and *Z. ramigera* (Zr) (27). Residues identical to those of the *Acinetobacter* β-ketothiolase are indicated by dots; gaps introduced into the alignment are indicated by dashed lines.
portions of all four coding segments on the basis of distances from putative promoter elements identified by sequence analysis (Fig. 4). Figure 5A shows the primer extension products identified upstream of the phoBAc coding region from the Acinetobacter isolate grown in ADM (limiting P) and ADM (lanes 1 and 2, respectively). A primer extension product (P3) corresponding to the major transcriptional start point was identified only in the Acinetobacter isolate grown in ADM (limiting P). TAATTT, which exhibits homology to the E. coli s70 −10 promoter consensus sequence, was identified 8 bp upstream of the P3 transcriptional start point. No sequence homologous to the −35 sequence was detected at the appropriate position upstream of this −10 region. Instead, we identified a sequence displaying similarity to an 18-bp Pho box consensus sequence shared by the regulatory regions of E. coli pho genes (Fig. 4A). Two additional primer extension products (P1 and P2) were also identified in the Acinetobacter sequence upstream of the phoBAc coding region, both of which were not affected by various phosphate concentrations.

Three potential transcriptional start points (corresponding to primer extension products P4, P5, and P6) were identified upstream of ORF1 from the Acinetobacter isolate grown in ADM (limiting P) (Fig. 5B). While the primer extension product corresponding to P6 is preceded by a potential −10 promoter consensus sequence (TTTCAAT), no −35 region was detected. However, 6 bp upstream of the −10 region, a second sequence displaying limited similarity to an E. coli pho box consensus sequence was identified (Fig. 4B, nucleotides 1824 to 1842). As distinct from P3, a faint primer extension product was observed from the Acinetobacter isolate grown in ADM. No sequence displaying similarity to an E. coli σ70 or σ24 promoter consensus sequence was identified upstream of either P5 or P6.

No primer extension products were observed immediately upstream of the phoCAc coding region. A single potential transcriptional start point (corresponding to primer extension product P7) was identified upstream of the phoCAc coding region from the Acinetobacter isolate grown in both ADM (limiting P) and ADM, suggesting that transcription resulting from an upstream promoter is not dependent on phosphate limitation (Fig. 5C, lanes 1 and 2). Further analysis of this region revealed the sequence ATATACAG−18 bp−TATATC, displaying similarity to an E. coli σ70 promoter consensus sequence (Fig. 4D). No potential Pho box sequences were identified in this region.

Transcriptional activation in E. coli wild-type and phoB mutant strains. Transcriptional activation from E. coli pho-regulated promoters has been shown to be dependent on the product of the PhoB gene, PhoB (16, 20). To determine whether the pho-activated promoters identified within the Acinetobacter PHA biosynthetic gene locus behaved similarly to these promoters, primer extensions were performed on RNA isolated from E. coli wild-type and phoB mutant strains containing pJKD1425. Figure 5A shows the results of these experiments for the segments upstream of the phoBAc coding region when these strains were grown in both limiting- and excess-phosphate media (lanes 3 to 6). A primer extension product corresponding to P3 was observed only in E. coli wild-type cells grown on limiting phosphate, suggesting a direct role for the PhoB protein in activating transcription from the upstream promoter. Primer extension products corresponding to P1 and P2 were also observed in E. coli. The reason these products...
appear more intense in *E. coli* wild-type cells is not apparent. Several other primer extension products not identified in *Acinetobacter* cells were also observed in *E. coli*.

Promoter extension experiments were also performed on the region upstream of ORF1, where the second *pha*-regulated promoter was identified. No primer extension products were observed from *E. coli* wild-type and *phaB* mutant strains grown in either limiting- or excess-phosphate medium (data not shown). These results suggest that the potential *pha* box identified in this region may not be recognized by the *E. coli* PhoB protein. No difference was observed in the transcription from the promoter identified upstream of the *phaA* coding region in *E. coli* grown in limiting- or excess-phosphate medium (Fig. 5C, lanes 3 and 4). This result is consistent with the primer extensions performed on *Acinetobacter* RNA.

**Analysis of promoter-CAT fusions.** To study the activities of the three promoter regions identified in the PHA biosynthetic gene locus, fusions were constructed with a promoterless CAT gene in plasmid pKK232-8. These plasmids were introduced into *E. coli* wild-type and *phaB* mutant strains, and CAT levels in these strains grown in limiting- and excess-phosphate media were measured (Fig. 1B). In the fusion with the upstream region of the *phaB* coding segment (pJKD1539), CAT levels were induced 20-fold by phosphate starvation. No induction was observed under the same conditions when pJKD1539 was introduced into the *E. coli* phoB mutant strain. Similar CAT levels were also obtained when only the *pha*-activated promoter region was present (pJKD1646). The CAT levels resulting from pJKD1646 and pJKD1539 revealed residual values in the *phaB* mutant strain and in the wild-type strain with excess phosphate. These results are in agreement with those of primer extension experiments and suggest the presence of an additional promoter(s) recognized in *E. coli* within these regions.

No promoter activity was manifested by the plasmid containing the region upstream of ORF1 (pJKD1537). This result reinforced the observed lack of recognition of this putative *pha*-activated promoter in *E. coli* observed in the primer extension experiments. The promoter activity resulting from the region upstream of the *phaA* coding segment (pJKD1536) was not significantly different in wild-type and *phaB* mutant strains grown in both limiting- and excess-phosphate media. This result supports the finding of a constitutive promoter upstream of the *phaA* coding region. This result is not surprising given that the enzyme encoded by this gene (β-ketothiolase) is involved in both PHA biosynthesis and degradation.

**DISCUSSION**

The PHA biosynthetic gene locus of *Acinetobacter* strain RA3849 was cloned and analyzed at the molecular level. Four adjacent open reading frames were identified, with predicted amino acid sequences of encoded β-ketothiolase (phaA), acetoacetyl-CoA reductase (phaB), and PHA synthase (phaC) showing strong similarity to the predicted amino acid sequences of corresponding proteins from other organisms. In addition, a fourth open reading frame (ORF1) was identified within this locus.

PHB accumulation has been shown to be induced by phosphate starvation in all *Acinetobacter* PHB-positive strains examined in our laboratory (31). The regulation of PHB biosynthesis has been studied extensively in *A. eutrophus* (33). The PHB biosynthetic genes are expressed constitutively and regulation at the enzyme level occurs via inhibition by free coenzyme A. In *Pseudomonas aeruginosa*, regulation occurs at the transcriptional level by a mechanism requiring RpoN during growth on gluconate but not on octanoate (34). In this work, we showed that transcription of the *Acinetobacter pha* locus (an operon of four genes) from the upstream *phaB* promoter is induced by phosphate limitation and that when a plasmid carrying the *pha* locus is transformed into *E. coli*, this induction requires the *E. coli* pho regulon activator PhoB. Therefore *Acinetobacter* spp. likely encodes a PhoB homolog.

Initial evidence for *pha* regulon control of the *pha* locus was
based on the finding of potential pho box consensus sequences upstream of the P3 and P6 transcription start points. Only the one upstream of P3 has been shown to be under pho regulon control, however. An alignment of this putative pho box sequence with the E. coli pho box consensus sequence is shown in Fig. 6. The sequence of the pho box upstream of P6 has only limited similarity to the E. coli pho box sequence and is probably meaningless.

A previous report has shown that the Acinetobacter PHA synthase gene (phaC_Ac) may be present in more than one copy (31). Therefore, it is possible that the primer extension products observed upstream of ORF1 in fact result from a second copy of this gene within the Acinetobacter genome. This would also explain the absence of primer extension products and promoter activity from this region in E. coli. Another explanation is that primer extension products P4, P5, and P6 are the result of premature termination of the reverse transcriptase. However, no potential secondary structures were identified in this region. Alternatively, the increased transcription observed from this region in the Acinetobacter genome under limiting-phosphate conditions may be associated with a general stress response of this organism. Cloning and purification of the putative Acinetobacter PhoB homolog may enable the region of interaction between this protein and the DNA to be determined by footprinting experiments.

Promoter-CAT fusions mapped the phosphate-activated promoter region upstream of the phaB_Ac coding segment to the region contained in the insert of pJKD1646. The primer extension products P1 and P2 were unaltered by various phosphate concentrations, suggesting they are associated with constitutive promoter elements. These constitutive promoters may serve to ensure a basal level of gene expression under all growth conditions, therefore permitting PHB accumulation under other forms of nutrient limitation (e.g., nitrogen or sulfur).

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REFERENCES


