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Phosphate Concentration Regulates Transcription of the *Acinetobacter* Polyhydroxyalkanoic Acid Biosynthetic Genes

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The polyhydroxyalkanoic acid (PHA) biosynthetic gene locus was cloned and characterized from an *Acinetobacter* sp. isolated from activated sludge. Nucleotide sequence analysis identified three clustered genes, $phaA_{Ac}$ (encoding a β-ketothiolase), $phaB_{Ac}$ (encoding an acetoacetyl coenzyme A reductase), and $phaC_{Ac}$ (encoding a PHA synthase). In addition, an open reading frame (ORF1) with potential to encode a 13-kDa protein was identified within this locus. The sequence of the putative translational product of ORF1 does not show significant similarity to any sequences in the database. A plasmid containing the *Acinetobacter pha* locus conferred the ability to accumulate poly-β-hydroxybutyrate on its *Escherichia coli* host. These genes appear to lie in an operon transcribed by two promoters upstream of $phaB_{Ac}$, an apparent constitutive promoter, and a second promoter induced by phosphate starvation and under *pho* regulon control. These as well as a number of additional potential transcription start points were identified by a combination of primer extension and promoter-chloramphenicol acetyltransferase gene fusion studies carried out in *Acinetobacter* or *E. coli* transformants.

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 enzyme level as a result of inhibition of the β-ketothiolase by free coenzyme A (33). The evidence presented in this report suggests an alternative mechanism for the regulation of PHB synthesis in *Acinetobacter* spp., which involves activation of the PHA 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 PHA 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* transformants 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 NaH_2PO_4 $2H_2O$ 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 DyeDeoxy Terminator Cycle Sequencing Kit (Applied

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Acinetobacter strain RA3849	PHB positive	31
E. coli		
CA8000	*thi*	3
G206	*thi phoB62*	17
Plasmids		
pKK232-8	Ap^r promoter selection vector	4
pUC19	Ap^r cloning vector	23
pJKD1226	5-kb fragment containing $phaC_{Ac}$ in pRK404	31
pJKD1341	4.4-kb fragment overlapping insert of pJKD1226 in pUC19	This study
pJKD1425	5.6-kb fragment containing PHA biosynthetic gene locus in pUC19	This study
pJKD1536	943-bp *Hin*dIII-*Dra*I $phaA_{Ac}$ upstream region in pKK232-8	This study
pJKD1537	659-bp *Hin*dII-*Eco*RI ORF1 upstream region in pKK232-8	This study
pJKD1539	1.15-kb *Hin*dIII-*Hin*dII $phaB_{Ac}$ upstream region in pKK232-8	This study
pJKD1646	201-bp PCR product from primers 1784 and 2013 containing *pho*-activated promoter from $phaB_{Ac}$ upstream region in pKK232-8	This study

Biosystems) and analyzed on an Applied Biosystems model 373A DNA sequencing system. Amplification of DNA by PCR was performed with an FTS-1 Thermal Sequencer (Corbett Research).

For construction of promoter-chloramphenicol acetyltransferase (CAT) gene fusions, fragments were generated by using appropriate restriction endonucleases, made blunt, and cloned into the *Sma*I site of pKK232-8. Restriction endonuclease mapping was used to confirm the correct orientation of inserts. For construction of pJKD1646, primers 1784 and 2013 were used to amplify a 201-bp PCR product, which was subsequently ligated into pUC19. This construct was sequenced to verify that no errors had occurred in the PCR and to determine the orientation of the insert and finally was directionally cloned into the *Bam*HI-*Hin*dIII sites of pKK232-8.

RNA preparation and Northern (RNA) blot analysis. Total RNA was isolated from *E. coli* cultures by the single-step method (7), using TRISOL (Gibco, BRL). Twenty-five micrograms of RNA was denatured and electrophoresed on a standard formaldehyde gel as described previously (29). RNA was transferred to nylon membranes, and hybridization was performed at high stringency, using a digoxigenin nonradioactive DNA labeling and detection kit (Boehringer Mannheim) as instructed by the manufacturer.

Primer extension analysis. Primer extension reactions were performed with a primer extension system (Promega) as instructed by the manufacturer. Oligonucleotide primers were labeled with [γ-^{32}P]dATP (Amersham) at the 5′ end by using polynucleotide kinase and hybridized to 50 μg of total RNA. After extension with reverse transcriptase, cDNA products were examined by electrophoresis through an 8% polyacrylamide gel. To map transcriptional start points, sequencing reactions were performed on the corresponding DNA by the dideoxy-chain method (30), using [α-^{35}S]dATP and a T7 sequencing kit (Pharmacia LKB) with the same primers used for the primer extension reactions.

Enzyme assays. Cell extracts of *E. coli* strains were prepared for enzyme assays by passaging through a French pressure cell (Aminco, Silver Spring, Md.). Protein concentrations were determined as described previously (19) with bovine serum albumin as the standard. CAT assays were performed with a CAT enzyme-linked immunosorbent assay kit (Boehringer Mannheim) as instructed by the manufacturer. Two protein extracts were assayed from each sample, and the A_{405} was measured with a Bio-Rad model 450 microplate reader. As a control to correct for differences in plasmid copy number, β-lactamase assays were performed on the same cell extracts as described previously (6).

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to the GenBank nucleotide sequence database under accession number L37761.

RESULTS

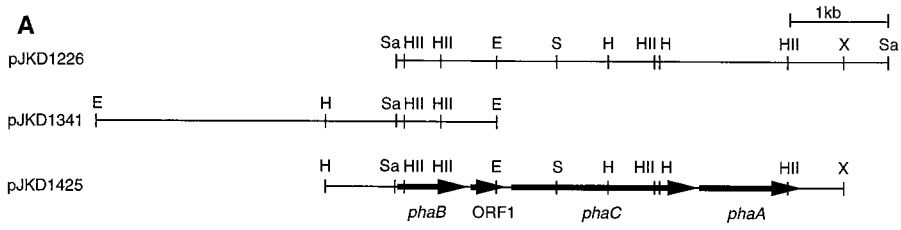
Identification of the *Acinetobacter* PHA biosynthetic gene locus. The *Acinetobacter* PHA synthase gene ($phaC_{Ac}$) was cloned by heterologous complementation in the PHB-negative mutant *A. eutrophus* PHB-4 as previously described (31). Nucleotide sequencing both upstream and downstream of $phaC_{Ac}$ on pJKD1226 revealed the presence of two other PHA biosynthetic genes, $phaB_{Ac}$ (encoding an acetoacetyl-CoA reductase) and $phaA_{Ac}$ (encoding a β-ketothiolase) (Fig. 1A). This plasmid did not confer the ability to accumulate PHB to its *E. coli* host, and subsequent sequence analysis revealed the promoter region to be absent.

To clone this upstream promoter region, a 4.4-kb *Eco*RI 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 *Hin*dIII-*Xba*I 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 *Hin*dIII-*Xba*I 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 acetoacetyl-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



B

	Units CAT/ unit β-lactamase (mean ± SD)			
	Wild-type		*phoB* mutant	
	Li Pi	Ex Pi	Li Pi	Ex Pi
pJKD1539	7.03 ± 0.09	0.35 ± 0.04	0.24 ± 0.06	0.29 ± 0.04
pJKD1646	9.30 ± 3.20	0.39 ± 0.08	0.23 ± 0.02	0.39 ± 0.03
pJKD1537	0.23 ± 0.03	0.39 ± 0.14	0.16 ± 0.01	0.19 ± 0.02
pJKD1536	1.09 ± 0.50	1.38 ± 0.38	0.30 ± 0.06	0.87 ± 0.07
pKK232-8	0.06 ± 0.03	0.07 ± 0.02	0.02 ± 0.01	0.01 ± 0.00

FIG. 1. (A) Physical map of the *Acinetobacter* strain RA3849 PHA biosynthetic gene locus reconstructed from two overlapping clones. The left-hand *Sau*3AI site of pJKD1226 maps at nucleotide 989 in Fig. 4A, which lies within the putative *pho* box consensus sequence identified upstream of the $phaB_{Ac}$ coding region. (B) Regions of the *Acinetobacter* strain RA3849 PHA biosynthetic gene locus cloned into the promoter selection vector pKK232-8 and corresponding CAT levels obtained when plasmids were introduced into *E. coli* wild-type and *phoB* 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, *Eco*RI; H, *Hin*dIII; HII, *Hin*dII; S, *Sph*I; Sa, *Sau*3AI; X, *Xba*I.

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′

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Ac   1   MSEQKVALVT GALGGIGSEI CRQLVTAGYK IIATVVPREE DREKQWLQSE GFQDSDVRFV
Ae   1    MT.RI.Y.. .GM....TA. .QR.AKD.FR VV.GCG.-NS P.REK..EQQ KALGF.FIAS
Cv   1     MARI.... .GI....TS. .TR.AKD.CT VV.NCH.S.A AAAEE..K.AR AAEGF.IAVF
Zr   1     MSR..... .GSR...AA. SIA.KA.... VA.SYAG-ND -------DAA KP----FKAE

Ac  61   LT------DL NNHEAATAAI QEAIAAEGRV DVLVNNAGIT RDATFKKMSY EQWSQVIDTN
Ae  59   EG------NV ADWDSTKT.F DKVKSEV.E. ...I...... ..VV.R..TR AD.DA.....
Cv  59   TA------.V SSFDDSARMV R.ITEQV.PI .I...C.... .RKT....EQ AH.EA..NV.
Zr  48   TGIAVYKW.V SSY..CVEG. AKVE.DL.PI .......... K..M.H..TP D..NA..N..

Ac 115   LKTLFTVTQP VFNKMLEQKS GRIVNISSVN GLKGQFGQAN YSASKAGIIG FTKALAQEGA
Ae 113   .TS..N..KQ .IDG.ADRGW .......... .Q......T. ..TA...LH. ..M.....V.
Cv 113   .NSV.N..RQ YWDG...RGF ...I...... .QR....... ...A...MH. ..M.......
Zr 108   .TG..NM.H. .WSG.RDRSF ........I. .Q...M.... ...A...DL. ..........

Ac 175   RSNICVNVVA PGYTATPMVT AMREDVI-KS IEAQIPLQRL AAPAEIAAAV MYLVSEHGAY
Ae 173   TKGVT..T.S ...I..D..K .I.Q..L-DK .V.T..VK.. GL.E...SIC AW.S..ESGF
Cv 173   SKGVT..TIS ...VE.A.TL ..ND..R-N. .ISG..MR.M .Q.N.....I AF.AGDESG.
Zr 168   AKG.T..AIC ...IG.E..R .IP.K.LNER .IP...VGLR GE.D...RI. VF.A.DEAGF

Ac 234   VTGETLSING GLYMH
Ae 232   S..ADF.L.. ..H.G
Cv 232   M..AN.PV.. ..F..
Zr 228   I..S.I.A.. .QFFV
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FIG. 2. Alignment of the amino acid sequence deduced from the nucleotide sequence of the *Acinetobacter* (Ac) gene $phaB_{Ac}$ (this study) with sequences of the acetoacetyl-CoA reductases of *A. eutrophus* (Ae) (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.

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Ac   1    MKDVVIVAA KRTAIG-SFL GSLASLSAPQ LGQTAIRAVL DS-ANV--KP EQVDQVIMGN
Ae   1    MT...VIS. A...V.-K.G ....KIP..E ..AVV.K.A. ER-.G.--.. ...SE....Q
Cv   1   MSENI...D. G.S...-T.G ...S....TE I.TAVLKGL. AR-TGL--A. ..I.E..L.Q
Zr   1   MSTPSIVI.S A...V.-..N .AF.NTP.HE ..A.V.S... ER-.G.--AA GE.NE..L.Q
Ec   1    MEQ....D. I..PM.R.KG .AFRNVR.ED .SAHLM.SL. ARNPAL--EA AAL.DIYW.C
Su   1   MSQN.Y..ST A..P..-..Q ...S.KT.VE ..AA.LKGA. AK-VPELDAS KDF.EI.F..

Ac  56   V-LTTGVGQN PARQAAIAAG IPVQVPASTL NVVCGSGLRA VHLAAQAIQC DEADIVVAGG
Ae  56   .-..A.S... .......K.. L.AM...M.I .K......K. .M...N..MA GD.E......
Cv  57   .-..A..... ....TTLH.. L.HS...M.I .K......K. ....M...A. GD....I...
Zr  57   .-.PA.E... ......MK.. V.QEAT.WGM .QL..... .A.GM.Q.AT GD.S.I....
Ec  58   .QQ.LEQ.F. I..N..LL.E V.HS...V.V .RL...SMQ. L.D..RM.MT GD.QACLV..
Su  59   .-.SANL..A ....V.LT.. LGNHIV.T.V .K..A.AMK. II.G..S.K. GN..V.....

Ac 115   QESMSQSAHY MQL-RNGQKM GNAQLVDSMV ADGLTDAYNQ YQMGITAENI VEKLGLNREE
Ae 115   ..N..AAP.V LPGS.D.FR. .D.K...T.I V...W.V... .H.......V AKEY.IT..A
Cv 116   .......S.V LPRS.D..R. .DWSMK.T.I V...W..F.N .H..T..... AQ.Y.FT..Q
Zr 116   M....MAP.C AH.A--.V.. .DFKMI.T.I K......FYG .H..T....V AKQWQ.S.DE
Ec 118   V.H.GH---- VPMSHGVDFH PGLS-RNVAK .A.M------ --..L...ML ARMH.IS..M
Su 118   C...TNAPY. .PAA.G.A.F .QTV.I.GVE R...N...DG LA..VH..KC ARDWDIT.DQ

Ac 174   QDQLALTSQQ RAAAAQAAGK FKDEIAVVSI PQRKGEPVVF AEDEYIKANT SLESLTKLRP
Ae 175   ..EF.VG..N K.E...K... .DE..VP.L. .....D..A. KT..FVRQGA T.D.MSG.K.
Cv 176   ..AF.AA... KTE...K..R .Q...IPIE. .....D.K.. DA..FPRHG. TA...G....
Zr 174   ..AF.VA..N K.E..KKD.R .....VPFIV KG...DIT.- DA....RHCA T.D.MA....
Ec 165   ..AF.AR.HA ..W..TQSAA ..N..IPTGG HDAD.VLKQ. NY..V.RPE. TV.A.AT...
Su 178   ..SF.IE.Y. KSQQS.KE.. .DN..VP.T. KGFR.K.DTQ VTNDEEP.RL HV.K.KSA.T

Ac 234   AF-KKD-GSV TAGNASGIND GAAAVLMMSA DKAAELGLKP LARIKGYAMS GIEPEIMGLG
Ae 235   ..-D.A-.T. ..A....L.. .....VV... A..K....T. ..T..S..NA .VD.KV..M.
Cv 236   ..-S..-... .......... ...M.VV.KE S..K..... M..LVAF.SA .VD.A...T.
Zr 233   ..-D.E-.T. ......L.. ....A.L..E AE.SRR.IQ. .G..VSW.TV .VD.KV..T.
Ec 225   ..DPVN-.M. ...TS.ALS. ....MLV..E SR.H..... R..VRSM.VV .CD.S...Y.
Su 238   V.-QREN.T. ..A...P... ....IILV.E RVLK.KN... ..IVK.WGEA AHL.ADFTWA

Ac 292   PVDAVKKTLN KAGWSLDQ-V DLIEANEAFA AQALGVAKEL GLDL---DKV NVNGGAIALG
Ae 293   ..P.S.RA.S R.E.TPQD-L ..M.I..... ....A.HQQM .W.T---S.. ........I.
Cv 294   .IP.STKC.E ....TPAD-L .......... ...MS.NQDM .W.---S.. ........I.
Zr 291   .IP.SR.A.E R...KIGD-L ..V..... ...CA.N.D. .W.P---SI. ........I.
Ec 284   ..P.S.LA.K ...L.ASD-I GVF.M..... ...I.PCI.D. ..IEQIDE.I .L........
Su 297   .SL..P.A.K H..IEDINS. .YF.F....S VVG.VNT.I. K..P---S.. ..Y...V...

Ac 348   HPIGASGCRI LVTLLHEMQR RDAKKGIATL CVGG-GMGVA LAVERD
Ae 349   .......... ........K. ......L.S. .I..-..... .....K
Cv 350   .......A.V .....Y...K ......L... .I..-.Q... .....M
Zr 347   .......A.. .N...F..K. .G.R..L... .I..-..... MCIESL
Ec 343   ..L.C..A.. ST...NL.E. K.VQF.L.DG ..S.L.Q.I. TVF..V
Su 354   ..L.C..A.V V....SIL.Q EGG.I.V.AI .N...-.GASS VVI.KL
```

FIG. 3. Alignment of the amino acid sequence deduced from the nucleotide sequence of the *Acinetobacter* (Ac) gene $phaA_{Ac}$ (this study) with sequences of the β-ketothiolases of *A. eutrophus* (Ae) (26), *C. vinosum* (Cv) (18), *Z. ramigera* (Zr) (25), *E. coli* (Ec) (38), and *S. uvarum* (Su) (9). Residues identical to those of the *Acinetobacter* β-ketothiolase are indicated by dots; gaps introduced into the alignment are indicated by dashed lines.

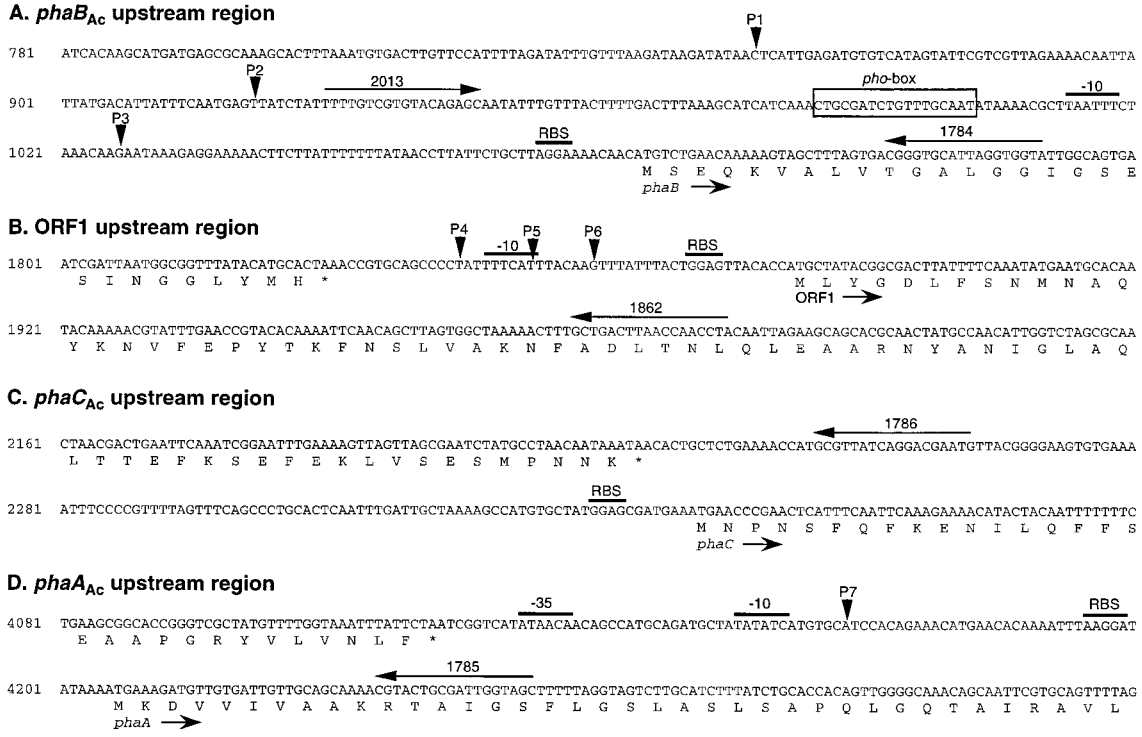

FIG. 4. Nucleotide sequences of the 5′ portions of the *phaB*$_{Ac}$ (A), ORF1 (B), *phaC*$_{Ac}$ (C), and *phaA*$_{Ac}$ (D) coding regions. The derived amino acid sequence of the relevant protein is indicated below the nucleotide sequence and specified by standard one-letter abbreviations. Putative promoter sequences, ribosome binding sites (RBS), and *pho* box are indicated. Potential transcription start sites corresponding to primer extension products P1 to P7 are indicated by arrows. Oligonucleotide primers used in this study are indicated by numbered arrows above the nucleotide sequence.

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 *phaB*$_{Ac}$ 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* σ^{70} −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 *phaB*$_{Ac}$ 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 (TTTCAT), 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* σ^{70} or σ^{54} promoter consensus sequence was identified upstream of either P5 or P6.

No primer extension products were observed immediately upstream of the *phaC*$_{Ac}$ coding region. A single potential transcriptional start point (corresponding to primer extension product P7) was identified upstream of the *phaA*$_{Ac}$ 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 ATAACA-18 bp-TATATC, displaying similarity to an *E. coli* σ^{70} 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 *phaB*$_{Ac}$ 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

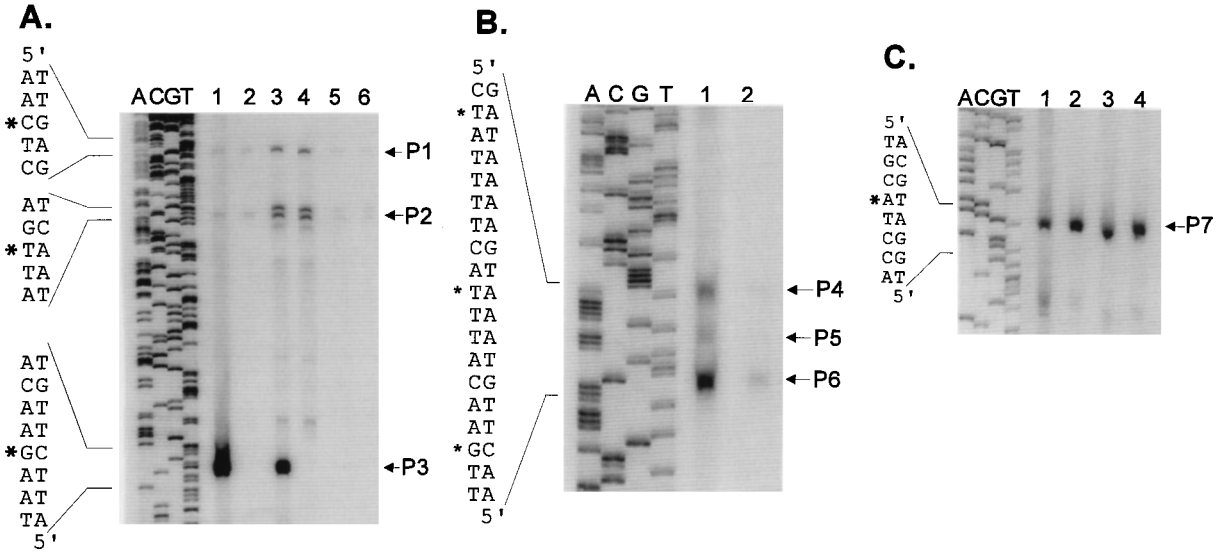

FIG. 5. Primer extension analysis of the *pha* locus. (A) $phaB_{Ac}$ upstream region. Lanes represent *Acinetobacter* strain RA3849 grown in ADM(limiting P) (lane 1) and ADM (lane 2), *E. coli* wild type containing pJKD1425 grown in TG limiting phosphate (lane 3) and excess phosphate (lane 4), and *E. coli phoB* mutant containing pJKD1425 grown in limiting phosphate (lane 5) and excess phosphate (lane 6). (B) ORF1 upstream region. Lanes represent *Acinetobacter* strain RA3849 grown in ADM(limiting P) (lane 1) and ADM (lane 2). (C) $phaA_{Ac}$ upstream region. Lanes represent *Acinetobacter* strain RA3849 grown in ADM(limiting P) (lane 1) and ADM (lane 2) and *E. coli* wild type containing pJKD1425 grown in limiting phosphate (lane 3) and excess phosphate (lane 4). Primer extension products are numbered P1 to P7. Nucleotide sequence analysis using the same primer for transcription start site mapping is shown in the lanes marked A, C, G, and T.

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.*

Primer extension experiments were also performed on the region upstream of ORF1, where the second *pho*-regulated promoter was identified. No primer extension products were observed from *E. coli* wild-type and *phoB* mutant strains grown in either limiting- or excess-phosphate medium (data not shown). These results suggest that the potential *pho* 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 $phaA_{Ac}$ 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 *phoB* 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 $phaB_{Ac}$ 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 *pho*-activated promoter region was present (pJKD1646). The CAT levels resulting from pJKD1646 and pJKD1539 revealed residual values in the *phoB* 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 *pho*-activated promoter in *E. coli* observed in the primer extension experiments. The promoter activity resulting from the region upstream of the $phaA_{Ac}$ coding segment (pJKD1536) was not significantly different in wild-type and *phoB* mutant strains grown in both limiting- and excess-phosphate media. This result supports the finding of a constitutive promoter upstream of the $phaA_{Ac}$ 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 $phaB_{Ac}$ 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 *pho* regulon control of the *pha* locus was

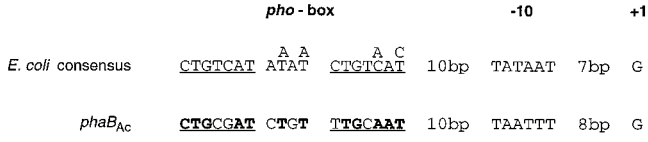

FIG. 6. Comparison of the *Acinetobacter pho* promoter region with that of the *E. coli* consensus sequence. The sequence of the *pho* box for $phaB_{Ac}$ is shown, together with the spacing to the −10 box of the proposed promoter and the transcription start point (+1). Nucleotides conserved between the proposed *Acinetobacter pho* box and the *E. coli* consensus sequence are indicated in boldface. Underlined bases represent the 7-bp repeat unit of the *pho* box.

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 putative *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

1. **Anderson, A. J., and E. A. Dawes.** 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. **54:**450–472.
2. **Bayly, R. C., A. Duncan, J. W. May, M. Schembri, A. Semertjis, G. Vasiliadis, and W. G. C. Raper.** 1991. Microbiological and genetic aspects of the synthesis of polyphosphate by species of *Acinetobacter*. Water Sci. Technol. **23:**747–754.
3. **Brickman, E., L. Soll, and J. Beckwith.** 1973. Genetic characterization of mutations which affect catabolite-sensitive operons in *Escherichia coli*, including deletions of the gene for adenyl cyclase. J. Bacteriol. **116:**582–587.
4. **Brosius, J.** 1984. Plasmid vectors for the selection of promoters. Gene **27:**151–160.
5. **Buchan, L.** 1983. Possible biological mechanism of phosphorus removal. Water Sci. Technol. **15:**87–103.
6. **Bush, K., and R. B. Sykes.** 1986. β-Lactamase (penicillinase, cephalosporinase), p. 280–285. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, vol. 3. VCH, Weinheim, Germany.
7. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
8. **Comeau, Y., K. J. Hall, R. E. W. Hancock, and W. K. Oldham.** 1986. Biochemical model for enhanced biological phosphorus removal. Water Res. **20:**1511–1521.
9. **Dequin, S., R. Gloeckler, C. J. Herbert, and F. Boutelet.** 1988. Cloning, sequencing and analysis of the yeast *S. uvarum ERG10* gene encoding acetoacetyl-CoA thiolase. Curr. Genet. **13:**471–478.
10. **Dienema, M. H., M. van Loosdrecht, and A. Scholten.** 1985. Some physiological characteristics of *Acinetobacter* spp. accumulating large amounts of phosphate. Water Sci. Technol. **17:**119–125.
11. **Echols, H., A. Garen, S. Garen, and A. Torriani.** 1961. Genetic control of repression of alkaline phosphatase in *E. coli*. J. Mol. Biol. **3:**425–438.
12. **Ellar, D., and D. G. Lundgren.** 1968. Morphology of poly-β-hydroxybutyrate granules. J. Mol. Biol. **35:**489–502.
13. **Griebel, R., Z. Smith, and J. M. Merrick.** 1968. Metabolism of poly-β-hydroxybutyrate. I. Purification, composition, and properties of native poly-β-hydroxybutyrate granules from *Bacillus megaterium*. Biochemistry **7:**3676–3681.
14. **Henrikson, S. D.** 1976. *Moraxella*, *Neisseria*, *Branhamella* and *Acinetobacter*. Annu. Rev. Microbiol. **30:**63–83.
15. **Juni, E.** 1972. Interspecies transformation of *Acinetobacter*. Genetic evidence for a ubiquitous genus. J. Bacteriol. **112:**917–931.
16. **Kasahara, M., K. Makino, M. Amemura, A. Nakata, and H. Shinagawa.** 1991. Dual regulation of the *ugp* operon by phosphate and carbon starvation at two interspaced promoters. J. Bacteriol. **173:**549–558.
17. **Kreuzer, K., C. Pratt, and A. Torriani.** 1975. Genetic analysis of regulatory mutants of alkaline phosphatase of *E. coli*. Genetics **81:**459–468.
18. **Liebergesell, M., and A. Steinbüchel.** 1992. Cloning and nucleotide sequences of genes relevant for biosynthesis of poly(3-hydroxybutyric acid) in *Chromatium vinosum* strain D. Eur. J. Biochem. **209:**135–150.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
20. **Makino, K., H. Shinagawa, M. Amemura, S. Kimura, and A. Nakata.** 1988. Regulation of the phosphate regulon of *Escherichia coli*: activation of *pstS* transcription by PhoB protein in vitro. J. Mol. Biol. **203:**85–95.
21. **Makino, K., H. Shinagawa, M. Amemura, and A. Nakata.** 1986. Nucleotide sequence of the *phoB* gene, the positive regulatory gene for the phosphate regulon of *Escherichia coli* K-12. J. Mol. Biol. **190:**37–44.
22. **Müller, H.-M., and D. Seebach.** 1993. Poly(hydroxyalkanoates): a fifth class of physiologically important organic biopolymers? Angew. Chem. Int. Ed. Engl. **32:**477–502.
23. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligonucleotide-directed mutagenesis. Gene **26:**101–106.
24. **Pieper-Fürst, U., M. H. Madkour, F. Mayer, and A. Steinbüchel.** 1994. Purification and characterization of a 14-kilodalton protein that is bound to the surface of polyhydroxyalkanoic acid granules in *Rhodococcus ruber*. J. Bacteriol. **176:**4328–4337.
25. **Peoples, O. P., S. Masamune, C. T. Walsh, and A. J. Sinskey.** 1987. Biosynthetic thiolase from *Zoogloea ramigera*. III. Isolation and characterization of the structural gene. J. Biol. Chem. **262:**97–102.
26. **Peoples, O. P., and A. J. Sinskey.** 1989. Poly-β-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Characterization of the genes encoding β-ketothiolase and acetoacetyl-CoA reductase. J. Biol. Chem. **264:**15293–15297.
27. **Peoples, O. P., and A. J. Sinskey.** 1989. Fine structural analysis of the *Zoogloea ramigera phbA-phbB* locus encoding β-ketothiolase and acetoacetyl-CoA reductase: nucleotide sequence of *phbB*. Mol. Microbiol. **3:**349–357.
28. **Rees, G. N., G. Vasiliadis, J. W. May, and R. C. Bayly.** 1993. Production of poly-β-hydroxybutyrate in *Acinetobacter* spp. isolated from activated sludge. Appl. Microbiol. Biotechnol. **38:**734–737.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
31. **Schembri, M. A., R. C. Bayly, and J. K. Davies.** 1994. Cloning and analysis of the polyhydroxyalkanoic acid synthase gene from an *Acinetobacter* sp.: evidence that the gene is both plasmid and chromosomally located. FEMS Microbiol. Lett. **118:**145–152.
32. **Steinbüchel, A., E. Hustede, M. Liebergesell, U. Pieper, A. Timm, and H. Valentin.** 1992. Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. FEMS Microbiol. Rev. **103:**217–230.
33. **Steinbüchel, A., and H. G. Schlegel.** 1991. Physiology and molecular genetics of poly(β-hydroxyalkanoic acid) synthesis in *Alcaligenes eutrophus*. Mol. Microbiol. **5:**535–542.
34. **Timm, A., and A. Steinbüchel.** 1992. Cloning and molecular analysis of the

poly(3-hydroxyalkanoic acid) gene locus of *Pseudomonas aeruginosa* PAO1. Eur. J. Biochem. **209:**15–30.

35. **Vanveen, H. W., T. Abee, G. J. J. Kortstee, W. N. Konings, and A. J. B. Zehnder.** 1993. Characterization of two phosphate transport systems in *Acinetobacter johnsonii* 210A. J. Bacteriol. **175:**200–206.
36. **Wanner, B. L.** 1987. Phosphate regulation of gene expression in *Escherichia coli*, p. 1326–1333. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
37. **Wanner, B. L.** 1993. Gene regulation by phosphate in enteric bacteria. J. Cell. Biochem. **51:**47–54.
38. **Yang, S.-U., X.-Y. H. Yang, G. Healy-louie, H. Schulz, and E. Marshall.** 1990. Nucleotide sequence of the *fadA* gene. Primary structure of 3-ketoacyl-coenzyme A thiolase from *Escherichia coli* and the structural organization of the *fadAB* operon. J. Biol. Chem. **265:**10424–10429.