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Molecular Analysis of the *phoH* Gene, Belonging to the Phosphate Regulon in *Escherichia coli*

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By making operon fusions with λp*lac*Mu53, we identified, cloned, and analyzed the *phoH* gene belonging to the phosphate (*pho*) regulon. We mapped the *phoH* gene at 23.6 min in the *Escherichia coli* genomic library (Y. Kohara, K. Akiyama, and K. Isono, Cell 50:495–508, 1987). Its nucleotide sequence revealed an open reading frame of 354 amino acids which contains sequences for nucleotide-binding motifs. From comparison of the DNA sequences, *phoH* was found to be identical to *psiH*, which had been identified as a phosphate starvation-inducible gene (W. W. Metcalf, P. M. Steed, and B. L. Wanner, J. Bacteriol. 172:3191–3200, 1990). The PhoH protein was overproduced by the T7 promoter system, identified as a protein of about 39 kDa, and purified. The amino-terminal amino acid sequence of the PhoH protein agreed with the one deduced from the DNA sequence. We demonstrated that PhoH has an ATP-binding activity by a photoaffinity labeling experiment. Two transcriptional initiation sites (P1 and P2) were identified by S1 nuclease mapping. The upstream P1 promoter contains a *pho* box, the conserved sequence shared by the *pho* regulon genes. The region containing the *pho* box was bound by PhoB protein, the transcriptional activator of the *pho* regulon, as revealed by footprinting. Regulation of *phoH* expression in vivo was studied by constructing plasmids containing transcriptional fusions of the *phoH* promoters with a promoterless gene for chloramphenicol acetyltransferase. Transcription from the P1 promoter required the *phoB* function and was induced by phosphate limitation, while transcription from the P2 promoter was independent of *phoB* and constitutive under tested conditions.

Escherichia coli responds to phosphate limitation by activating transcription of genes belonging to the phosphate (*pho*) regulon, whose products are involved in the transport and use of various forms of combined phosphates or free phosphate (31, 36, 38). The *phoB* gene product is the transcriptional activator for the *pho* regulon. Responding to phosphate limitation, the *phoR* gene product undergoes autophosphorylation with ATP and acts as a protein kinase for the activator protein PhoB (22). Phosphorylated PhoB binds to the *pho* box, a consensus sequence of the *pho* regulon promoters, and activates transcription with RNA polymerase containing the major sigma factor, σ^{70} (23). The genes in the *pho* regulon include *phoA*, *phoE*, and the genes in the *pst*, *ugp*, and *phn* operons.

Wanner et al. isolated 55 phosphate starvation-inducible (*psi*) promoters, which include the *pho* promoters, by constructing operon fusions to *lacZ* using Mu dl(Ap^r *lac*) (39, 40). We made similar fusions using λp*lac*Mu53 (21). One such mutant, SE5008 (*phoH*::λp*lac*Mu53), was characterized. The complete *phoH* gene was cloned and characterized. We identified two promoters of *phoH*, one inducible by phosphate limitation and the other constitutive. PhoH protein as deduced from the DNA sequence of the gene has putative nucleotide-binding motifs, and indeed the purified PhoH protein had an ATP-binding activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. *E. coli* SE5000 [F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301 recA56*] was used for the isolation of in vivo operon-fusion genes with phage λp*lac*Mu53 (3). *E. coli* CJ236 [*dut-1 ung-1 thi-1 relA1*/pCJ105 (F′ Cam^r)] was obtained from Takara Shuzo (Kyoto, Japan) and used for site-directed mutagenesis by the Kunkel method (18). *E. coli* JM103 (26) was used as a host for M13 phages. *E. coli* BL21 (DE3) (33), which carries the inducible T7 RNA polymerase gene on the chromosome, was used for hyperexpression of the *phoH* gene under the T7 promoter. CSH66Δ*lac* was obtained by curing the lysogenized λ (*c*I857*S7plac5 i*$^-$ *z*$^+$ *y*$^-$) phage of CSH66 (28) by heat induction. CSH66Δ*lac* (*phoH*::λp*lac*Mu53) was constructed by transduction with P1 grown on SE5008 (*phoH*::λp*lac*Mu53). pUC9 (37) and pUC19 (41) were used as the cloning vectors. Phages M13mp18 and M13mp19 (41) were used for subcloning and sequencing of the *phoH* region. pMC1403 (5) and pKK232-8 (4), which carry the promoterless *lacZ* and *cat* genes, respectively, as reporter genes, were used as the cloning vectors. Phages M13mp18 and M13mp19 (41) were used for assaying promoter activities. A T7 promoter-containing plasmid, pT7-7 (34), was used to construct the overproducing plasmid. Bacteriophage λp*lac*Mu53 [*imm*λ*′trp′-′lacZ*$^+$ *lacY*$^+$ *lacA′ ′uvrD′ Xho*::*kan* Mu(*c*Its62*ner*$^+$ *A*$^+$ *′S*)] was used for constructing operon fusions in vivo (3). SE5000 and λp*lac*Mu53 were kindly supplied by E. Bremer. The lambda phage clones of the ordered genomic library of *E. coli* were kindly supplied by Y. Kohara.

Media and enzyme assays. Luria-Bertani (LB) liquid medium, Luria-Bertani agar plates, and Tris-glucose (TG) medium supplemented with excess phosphate (TGHP) or limiting phosphate (TGLP) were described previously (2). The media used for the routine preparation of M13 phages were described previously (1). To detect β-galactosidase (β-Gal) activity of the colony, 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml was added to agar plates (28). The activities of chloramphenicol acetyltrans-

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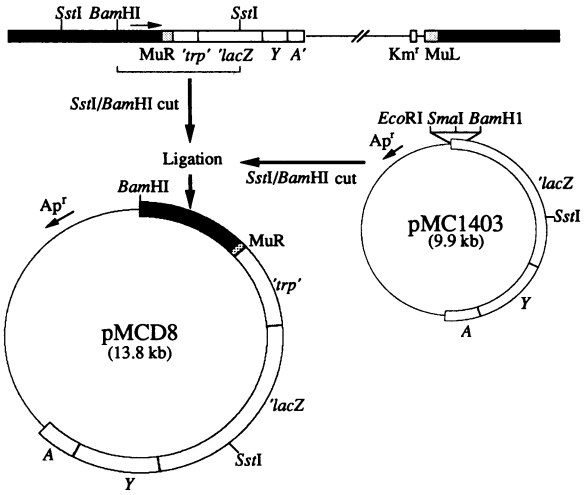

FIG. 1. Strategy for cloning a chromosomal fragment containing the fusion junction into a plasmid. Black boxes are *E. coli* chromosomal DNA; thin lines are lambda or vector plasmid DNA; dotted boxes are Mu sequences; and open boxes represent *'trp'-'lacZYA'*. The arrow above the map indicates the orientation of the gene with the λ*plac*Mu53 insertion. Plasmid pMCD8 contained the *phoH'*-MuR-*'trp'-'lacZYA* fusion encoding active β-Gal. Ap^r and Km^r represent ampicillin and kanamycin resistance genes, respectively.

ferase (CAT) and β-Gal in the cultured cells were assayed as described previously (16, 28).

DNA manipulation. Standard recombinant DNA methods were used (6, 25). The DNA sequences of the *phoH* region were analyzed by the dideoxy-chain termination method (30). DNA fragments to be sequenced were cloned into M13mp18 or M13mp19 phages. A series of phage clones that contained various deletions from one end of the cloned DNA fragments was prepared by the method of Hong (12). The DNA fragments with upstream deletions of the *phoH* regulatory region were prepared by exonuclease III and mung bean nuclease treatment (8). Restriction enzymes, exonucleases III, mung bean nuclease, phage T4 DNA ligase and polymerase, and M13 DNA sequencing kits were purchased from Takara Shuzo.

Methylation protection and analysis of transcripts. PhoB binding to the *phoH* regulatory region was analyzed by DNA methylation footprinting, and the transcription start sites were analyzed by S1 nuclease mapping of the in vivo transcripts, as described previously (22, 23). Purification of PhoB and PhoR1084 proteins and phosphorylation of PhoB by PhoR1084 were done as described previously (22).

Site-directed mutagenesis. The synthetic oligonucleotide 5′ CGTTACCATATGCGATCTCC 3′ (positions 1040 to 1021) was used to introduce a unique *Nde*I site at the translational start codon of *phoH*. A 1.4-kb blunt-ended *Apa*LI-*Sac*I fragment with the intact *phoH* gene (see Fig. 2B) was subcloned into the *Sma*I site of M13mp18, and the region containing the initiation codon ATG was converted to an *Nde*I site to make M13*phoH1*. Plasmid pT7-7H containing *phoH* was constructed by subcloning the *Nde*I-*Hin*dIII fragment from M13*phoH1* into pT7-7 digested with *Nde*I and *Hin*dIII.

Overproduction and purification of PhoH. *E. coli* BL21 (DE3) carrying plasmid pT7-7H was grown at 37°C to mid-exponential phase in 20 ml of Luria-Bertani medium

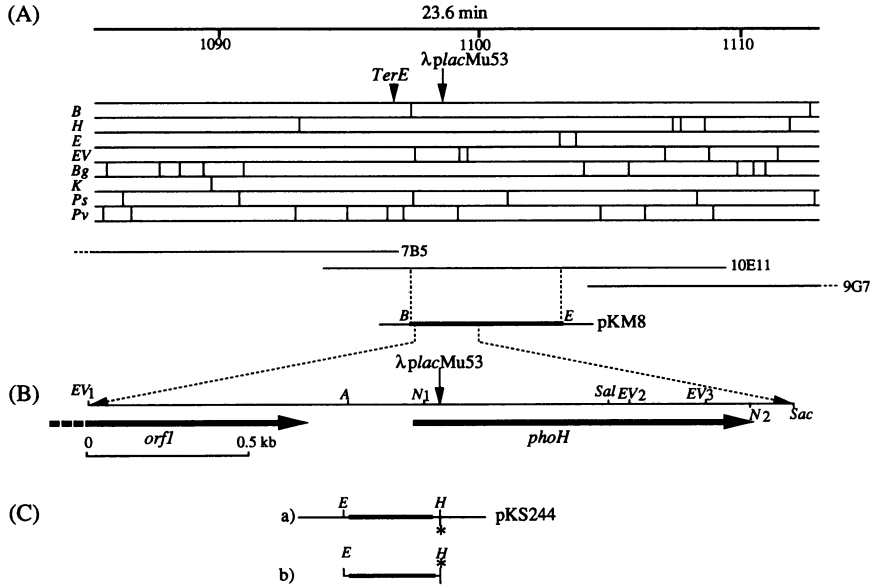

FIG. 2. Restriction map near the *phoH* gene. (A) Restriction map of the λ clone 10E11, as described by Kohara et al. (17). The vertical arrow indicates the λ*plac*Mu53 insertion site in SE5008. *terE* is shown by an arrowhead (9). The 6.1-kb *Bam*HI-*Eco*RI fragment (thick line) of clone 10E11 was subcloned into pUC9 (thin line) to make pKM8. (B) Enlarged restriction map of the *phoH* region. The horizontal arrows show the directions of transcription. (C) Restriction fragments carried on pKS244 were used for S1 mapping and methylation protection experiments. The portions of DNA derived from the *Apa*LI-*Nsp*I$_1$ chromosomal fragment and vector pUC19 are indicated by thick and thin lines, respectively. a) An *Eco*RI-*Hin*dIII fragment (about 300 bp) labeled with [γ-^{32}P]ATP by T4 DNA kinase at the *Hin*dIII end (*) was used as the sense strand probe for the S1 mapping and methylation protection experiments. b) An *Eco*RI-*Hin*dIII fragment (about 300 bp) labeled with [α-^{32}P]dCTP by Klenow fragment at the *Hin*dIII end (*) was used as the antisense strand probe for the methylation protection experiment. *B*, *Bam*HI; *H*, *Hin*dIII; *E*, *Eco*RI; *EV*, *Eco*RV; *Bg*, *Bgl*I; *K*, *Kpn*I; *Ps*, *Pst*I; *Pv*, *Pvu*II; *A*, *Apa*LI; *N*, *Nsp*I; *Sac*, *Sac*I; *Sal*, *Sal*I.

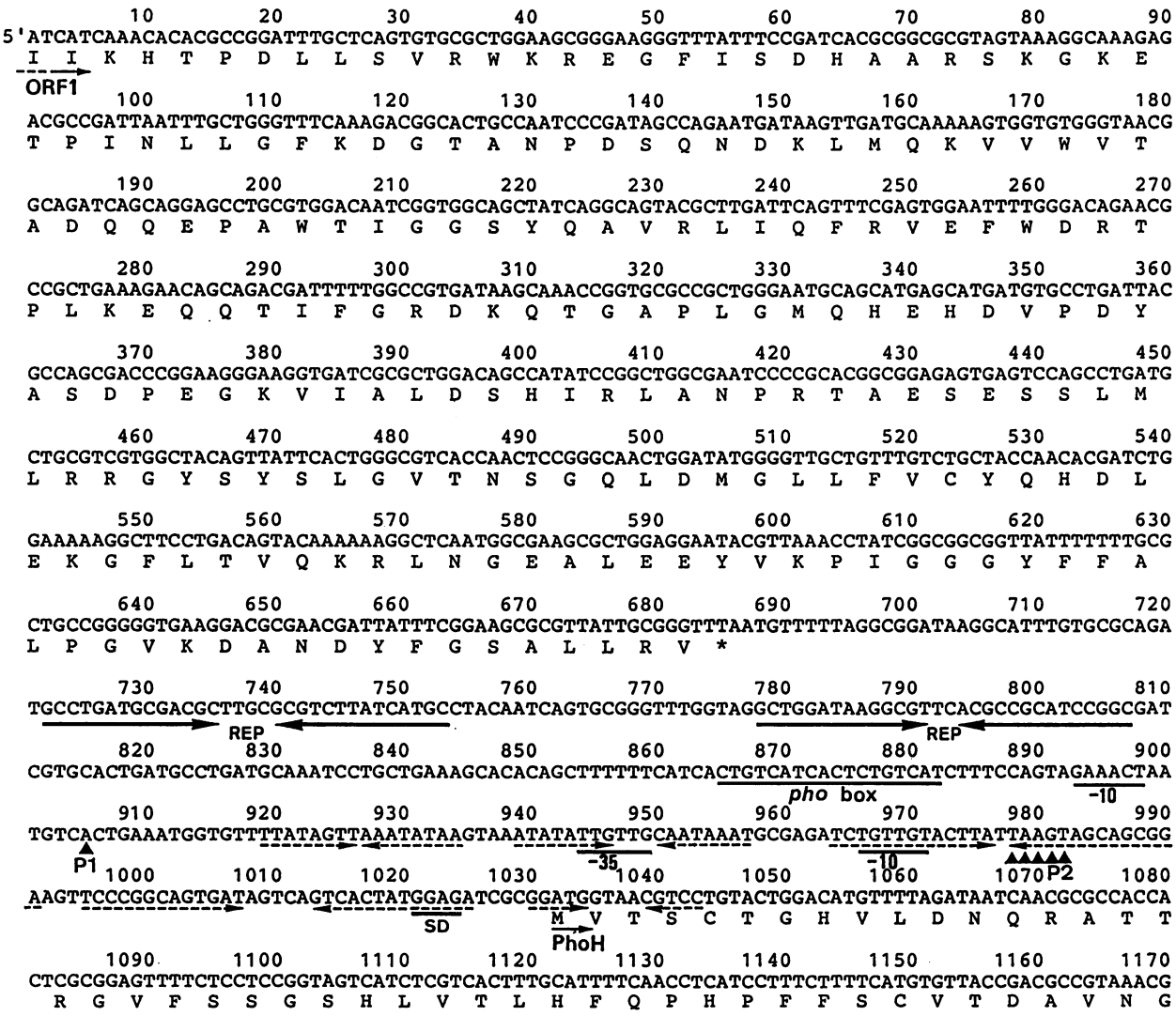

FIG. 3. Sequence of the *phoH* gene. Convergent arrows indicate repetitive-extracistronic-palindromic (REP) sequences. The *pho* box, −35, −10, and ribosome-binding (Shine-Dalgarno [SD]) sequences are indicated. The transcription initiation sites as determined by S1 mapping are indicated with triangles labeled P1 and P2. Dashed arrows show a potential stem-loop structure. Underlining indicates nucleotide-binding motifs. Convergent arrows between nucleotides 2111 and 2135 indicate a putative Rho-independent transcriptional terminator. Vertical arrows indicate the locations of *psiH45*, *psiH14*, and *psiH16* junctions (27, 32a).

containing 150 μg of ampicillin per ml. Cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. Cells were harvested and stored at −70°C. PhoH formed inclusion bodies in the overproducing strain. The inclusion bodies were purified as described by Igarashi and Ishihama (13) and solubilized with 0.1 ml of 6 M guanidine-HCl in 10 mM Tris-HCl (pH 8.0 at 4°C)–5% glycerol–0.1 mM EDTA–0.1 mM dithiothreitol. The solution was centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant was used for photoaffinity labeling. PhoH protein was purified to 90% homogeneity as judged from the Coomassie-stained gel. Strain BL21 (DE3) carrying the vector plasmid pT7-7 was grown, and the cell extract was processed in the same way as described above. This sample, which should contain much less PhoH protein, was used as a control for the photoaffinity labeling experiment.

Analysis of amino acid sequence of purified PhoH. The amino-terminal amino acid sequence of the purified PhoH protein was determined by automated Edam degradation with an Applied Biosystems 473A protein sequencer.

Photoaffinity labeling. Photoaffinity labeling of PhoH with a radioactive ATP analog, [γ-^{32}P]8N_3ATP, was done as described by Hobson et al. (11). Samples (0.2 μl) solubilized in 6 M guanidine-HCl were added with photoaffinity labeling buffer (10 mM Tris-HCl [pH 7.4], 5 mM $CaCl_2$, 10 mM $MgCl_2$) containing 4.5 μM 8-azido-[γ-^{32}P]ATP ([γ-^{32}P]8N_3ATP) (1 μCi; 1 Ci = 37 GBq) in a final volume of 20 μl. Samples were then mixed, incubated for 5 min on ice, and irradiated with a germicidal UV lamp (254 nm) at a distance of 2.5 cm for 2.5 min. The samples were subjected to electrophoresis in a sodium dodecyl sulfate–11.7% polyacrylamide gel (SDS-PAGE) as described previously (20). The gel was stained with Coomassie brilliant blue and subjected to autoradiography.

Radioisotopes and DNA hybridization. [α-^{32}P]dCTP (>400 Ci/mmol) and [γ-^{32}P]ATP (>5,000 Ci/mmol) were purchased

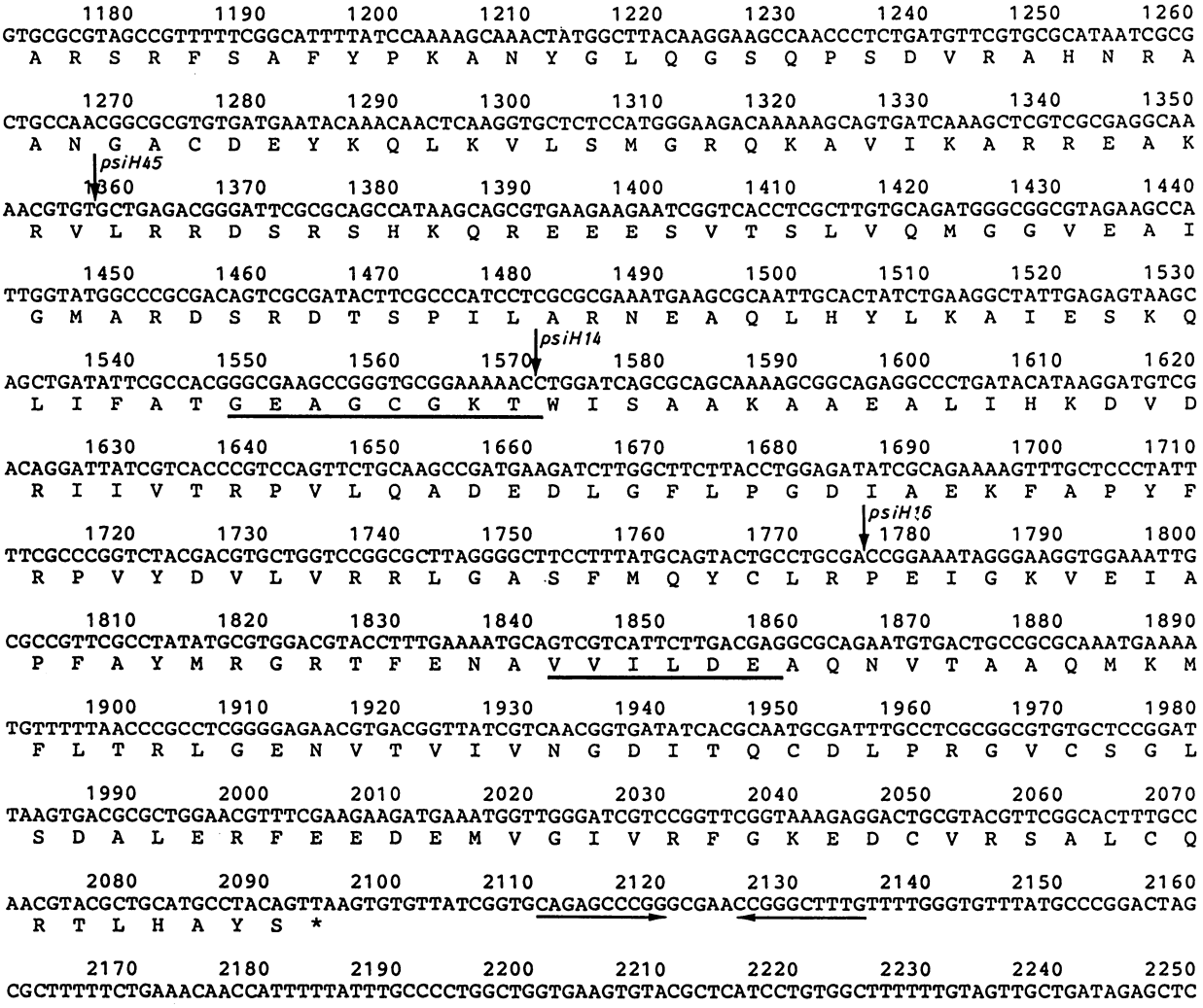

FIG. 3—*Continued.*

from Amersham Corp. DNA probes for Southern hybridization were labeled by nick translation (29) and used for plaque and Southern hybridization experiments (32).

Nucleotide sequence accession number. The nucleotide sequence of the *phoH* region (2,250 bp) has been submitted to the DDBJ, GenBank, and EMBL nucleotide sequence data bases under accession number D90448.

RESULTS

Cloning of DNA fragment carrying the functional *phoH* promoter region. SE5008 (*phoH*::λp*lac*Mu53) was strongly Lac$^+$ (dark blue) on TGLP agar and only weakly Lac$^+$ (pale blue) on TGHP agar media containing X-Gal and kanamycin. The β-Gal activities of SE5008 were 224 and 48 U, as defined by Miller (28), in phosphate-limited medium and phosphate-excess medium, respectively.

We cloned the DNA fragment containing the *phoH'-'lacZ* operon fusion as an *Sst*I or an *Sst*I-*Bam*HI fragment (Fig. 1). The λp*lac*Mu53 phage has an *Sst*I site 3.4 kb from MuR, which forms the fusion junction (27). Chromosomal DNA digested with *Sst*I or with *Bam*HI and *Sst*I enzymes revealed a single band by Southern hybridization with the *Bam*HI-*Sst*I fragment of pMC1403 (5) as the probe (Fig. 1). The 5.0-kb *Bam*HI-*Sst*I fragment that hybridized was electroeluted and ligated with *Bam*HI-*Sst*I-digested pMC1403 to reconstruct a functional *lacZ* gene (Fig. 1). SE5000 cells were transformed with the ligated DNA, and ampicillin-resistant (Ap^r) and Lac$^+$ transformants were selected. pMCD8, which carried the reconstructed *lacZ* gene, contained a 5.0-kb *Bam*HI-*Sst*I fragment of the chromosomal DNA of SE5008 (Fig. 1). The β-Gal activity of SE5000 cells carrying pMCD8 was induced by P_i starvation (data not shown), suggesting that it carried the functional *phoH* promoter fused with *lacZ*.

Identification of the phage integration site on the chromosome and cloning of the *phoH* locus. To clone the intact *phoH* gene, the *Bam*HI-*Sst*I fragment from pMCD8 was hybridized with the DNAs of lambda phage clones of the ordered genomic library of *E. coli* constructed by Kohara et al. (17). The *Bam*HI-*Sst*I fragment derived from pMCD8 hybridized with clone 10E11, which contains the chromosomal fragment located at 23.6 min (Fig. 2A). As expected, the DNA fragment also hybridized with clones 10A6, 7H10, 14C4, and 4F1, which contained *lacZ* or *trp* DNA present in the vector. The size of the cloned fragment indicated that λp*lac*Mu53

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PhoH        173 G E A G C G K T 180- - -271 V V I L D E 276
Consensus       G - - G S/C G K T/S  - - -   h h h h D E
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FIG. 4. Comparison of PhoH amino acid sequences with a consensus of nucleotide-binding proteins. h in the consensus sequence indicates a conserved hydrophobic residue.

was integrated about 1.6 kb from the *Bam*HI site (Fig. 2A), and the transcriptional direction of the *phoH* is clockwise. The 6.1-kb *Bam*HI-*Eco*RI fragment of clone 10E11 was subcloned into pUC9, yielding pKM8 (Fig. 2A).

Nucleotide sequence of the *phoH* region. Figure 2 shows the physical organization of the *phoH* locus. The 2.2-kb *Eco*RV$_1$-*Sac*I region (Fig. 2B) on pKM8 containing the *phoH* region was analyzed for both strands by the dideoxy-chain termination method with overlapping segments.

On the basis of the DNA sequence, the 2,250-bp fragment contains the carboxyl end of *orf1* in addition to the complete *phoH* gene sequence (Fig. 3). Two repetitive-extracistronic-palindromic sequences (7) were found between *orf1* and *phoH* (Fig. 3). The calculated M_r of PhoH is 39,263, with 354 amino acids. A putative binding site for the PhoB protein (*pho* box) is found upstream of the *phoH* coding region (Fig. 3). The space between the *pho* box and the −10 sequence of the *pho* promoters has been always 10 bp (21, 24). Downstream of the putative *pho* box with a 10-bp space is found the sequence gAaAcT that is similar to the consensus −10 sequence of many promoters. A typical ribosome-binding sequence, GGAG, is located at nucleotides 1021 to 1024. Downstream of *phoH*, a potential stem-loop structure with a calculated free energy of −21.8 kcal/mol (35) (1 cal = 4.184 J)
is found between nucleotides 2111 and 2135, followed by four T residues, which could form a Rho-independent transcriptional terminator.

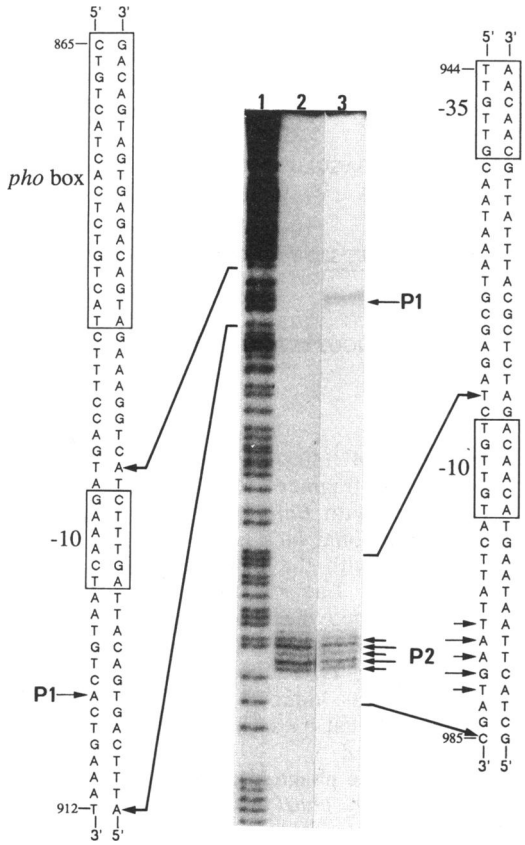

FIG. 5. S1 nuclease mapping of the transcription initiation sites of *phoH* in vivo. The end-labeled probe in Fig. 2C was hybridized to mRNA extracted from wild-type ANCK10/pKC244 cells (lane 2) and *pho* constitutive ANCC75/pKC244 cells (lane 3) grown in T broth. Lane 1 shows G+A ladders of Maxam-Gilbert sequencing of the probe DNA. The nucleotide sequences of the probe near the initiation sites are shown on both sides. The 5′ ends of the *phoH* transcripts are shown by arrows. The boxed sequences indicate the *pho* box, −35, and −10 regions of the promoter.

PhoH has nucleotide-binding motifs. Computer analysis was done with ORF1 and PhoH to the NBRF and SWISSPROT protein data bases and the translated GenBank and EMBL data bases. ORF1 revealed no statistically significant similarity to any protein. PhoH contains two highly conserved regions (Fig. 3 and 4), which may form a nucleotide-binding pocket of nucleotide-binding proteins (10). No other protein exhibited significant homology with PhoH.

mRNA start site of *phoH*. The *phoH* promoter was cloned into the pUC19 derivative as an *Apa*LI-*Nsp*I$_1$ fragment to make pKS244 (Fig. 2C). This plasmid was introduced into the wild-type strain ANCK10 and the *pho* constitutive strain ANCC75 (*pstS164*). mRNA was prepared from these strains after growth in T broth, which contains excess phosphate. The 5′ ends of the *phoH* gene transcripts were mapped (Fig. 5). Two *phoH* transcripts were detected in ANCC75/pKS244, while only one transcript was detected in ANCK10/pKS244. The longer transcript, denoted P1, initiated at nucleotide 905, was detected in ANCC75/pKS244 cells but not in ANCK10/pKS244 cells. Seven base pairs upstream from this transcription initiation site, a DNA sequence similar to the consensus sequence for the −10 region of many promoters exists, and there is a consensus *pho* box further upstream with a 10-bp space from the −10 sequence (Fig. 5). The shorter transcripts from the promoter designated P2 were initiated near nucleotide 980, which was found in ANCK10/pKS244 and ANCC75/pKS244 cells (Fig. 5), showing that the P2 promoter is constitutively expressed. We assigned the −10 region (TgTtgT) to nucleotides 966 to 971 and the −35 region (TTGttg) 17 bp upstream from the −10 region. Although the P1 band is weaker than the P2 bands in Fig. 5, which apparently contradicts the results of the analysis of the two promoter activities (see below), quantitative comparison of mRNA levels by S1 mapping is difficult because of the unstable nature of prokaryotic mRNA.

Functional analysis of the *phoH* promoters in vivo. To define the functional promoter region in vivo, we constructed plasmids with a series of upstream deletions of *phoH*. The *Apa*LI-*Nsp*I$_1$ region of pKS244 containing the *phoH* promoter (Fig. 2B and C) was recloned by using the flanking restriction sites *Bam*HI and *Hin*dIII in pKS244 into pKK232-8, and the resultant plasmid, pKC244, was used for the construction of a series of upstream deletions (Fig. 6). The plasmids that carry the promoterless *cat* gene fused downstream of these deletion fragments were introduced into ANCK10 (wild type) or ANCH1 [Δ(*phoB-phoR*)]. The promoter activity of the DNA fragments with the upstream deletions was measured by monitoring the CAT activity of the extracts of the cells carrying the plasmids grown in excess- or limited-phosphate medium. The endpoints of the deletions and their promoter activities are shown in Fig. 6.

The CAT activities of the ANCK10 cells with pKC5, pKC8, pKC13, or pKC28 were about fivefold less than that

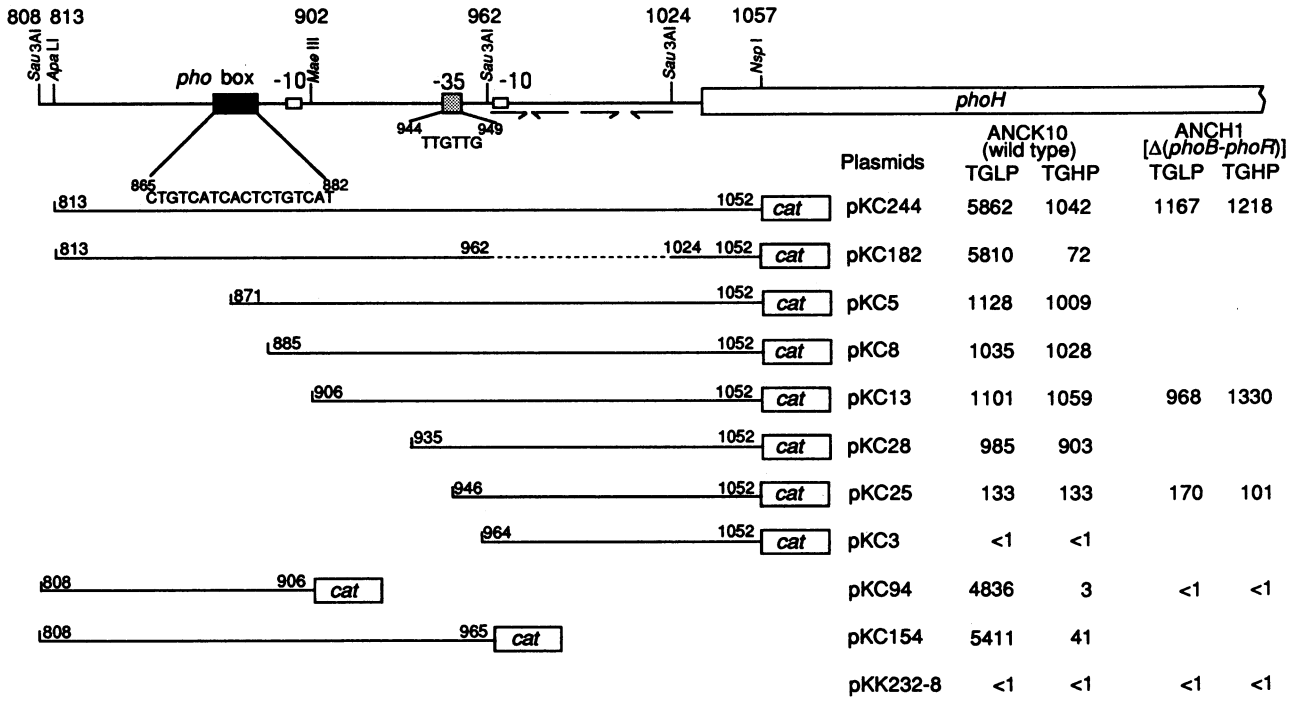

FIG. 6. Promoter activities of the *phoH* regions with various deletions. The *pho* box, −35, and −10 regions are shown by black, shaded, and white boxes, respectively. The sequences that may form a stem-loop structure are indicated by convergent half-arrows. The dotted line indicates a deletion. The CAT activities in ANCK10 or ANCH1 [Δ(*phoB-phoR*)] carrying the plasmids containing the indicated DNA fragments grown in TGHP or TGLP medium are shown on the right. CAT activities are expressed in nanomoles of 5-thio-2-nitrobenzoate liberated per minute per optical density unit of the cell culture at 450 nm (16).

of the cells with pKC244 containing the largest promoter region under limited-phosphate conditions, but they were similar under excess-phosphate conditions. The cells with pKC94 or pKC154 showed the same CAT activity under limited-phosphate conditions as the cells with pKC244, but they showed greatly reduced activity under excess-phosphate conditions. These results suggest that the sequence near nucleotide 871 is essential to the functioning of the P1 promoter under limited-phosphate conditions. This region has the sequence CTGTCATcAcTCTGTCAT, which is very similar to the consensus sequence of the *pho* box that has the 7-bp direct repeats flanking a 4-bp A(T)-rich sequence (24) (Fig. 7). The CAT activity of ANCH1 [Δ(*phoB-phoR*)] cells containing pKC244 was about fivefold less than that of ANCK10 cells containing the same fusion genes under

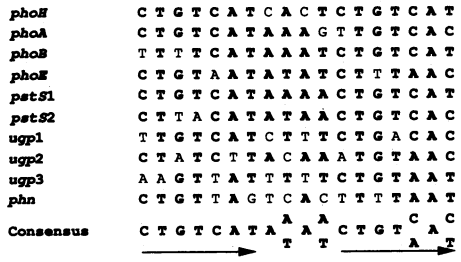

FIG. 7. Comparison of the *pho* box sequence of *phoH* with other *pho* box sequences. The nucleotides in bold letters are conserved among the majority of the *pho* boxes. The 7-bp direct repeats are indicated by arrows. References: *phoA*, *phoB*, *phoE*, *pstS1*, and *pstS2* (24); *ugp1*, *ugp2*, and *ugp3* (15); *phn* (21).

limited-phosphate conditions, but these cells showed the same activity under excess-phosphate conditions. These results suggest that the promoter P1 is activated by PhoB, and the CAT activities in ANCH1 reflect the activity of the promoter P2, which is PhoB independent. The CAT activities of ANCK10 and ANCH1 [Δ(*phoB-phoR*)] cells containing pKC25, which lacks the putative *pho* promoter and part of the −35 region of the putative P2 promoter, were low under excess- and limited-phosphate conditions (Fig. 6). No activity was observed with the fusion in pKC3, which lacks both promoters, under these genetic and nutrient conditions. Therefore, the upstream endpoint in pKC25 is essential to the P2 promoter that is active under the excess-phosphate conditions. The promoter activity of pKC182, which is deleted of the *Sau*3AI fragment containing the −10 sequence of the P2 promoter and two inverted repeat sequences (Fig. 3 and 6), was reduced only under excess-phosphate conditions. Hence, the −10 sequence is functional for the P2 promoter that is active under excess-phosphate conditions.

PhoB protein binding site. We examined the binding of PhoB to the regulatory region by methylation protection experiments (Fig. 8). Methylation of G at nucleotides 867 and 878 on the antisense strand and at 869 and 880 on the sense strand of the *pho* box region was much reduced by phosphorylated PhoB. Methylation protection of G at the same positions of the *pho* box has also been reported for the *phoB*, *pstS*, and *ugp* promoters (15, 22, 23). Therefore, these Gs may be very important for interaction with phosphorylated PhoB.

Overproduction and purification of PhoH. The *phoH* gene with a unique *Nde*I site at the ATG translational start site was made by site-directed mutagenesis; this gene was cloned

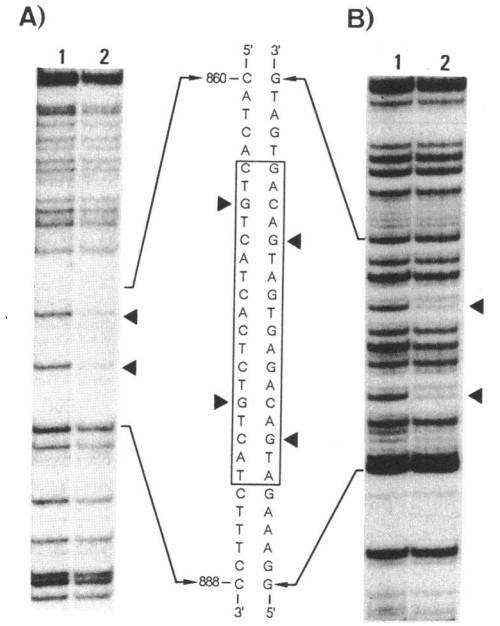

FIG. 8. Protection of the *phoH* promoter region by phosphorylated PhoB protein from methylation by dimethyl sulfate. The antisense strand (A) and the sense strand (B) probes were the *Eco*RI-*Hin*dIII fragments labeled with [α-^{32}P]dCTP and [γ-^{32}P]ATP, respectively, which are shown in Fig. 2C. The DNA bases markedly protected from methylation are marked with arrowheads. The labeled DNA was treated with dimethyl sulfate in the absence (lanes 1) or presence (lanes 2) of phosphorylated PhoB and then subjected to the Maxam-Gilbert G>A reaction. The sequence of the *phoH* regulatory region is in the middle. The *pho* box is boxed.

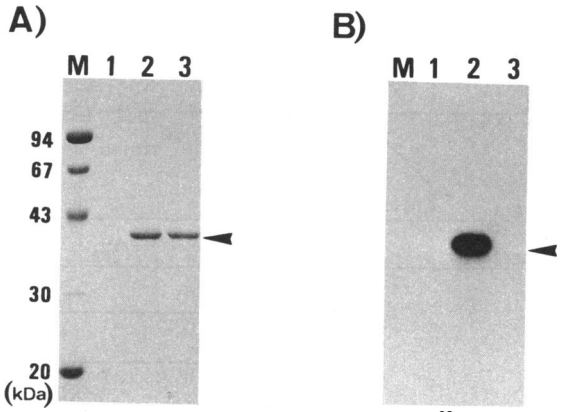

FIG. 9. Photoaffinity labeling of PhoH with [γ-^{32}P]8N_3ATP. (A) Samples were subjected to SDS–11.7% PAGE and stained with Coomassie brilliant blue. Lanes: M, molecular weight markers (top to bottom: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor); 1, fractionated sample corresponding to the PhoH fraction from the strain carrying vector plasmid pT7-7; 2, fractionated PhoH prepared from the strain carrying the PhoH-overproducing plasmid and subjected to UV photolabeling; 3, fractionated PhoH mixed with [γ-^{32}P]8N_3ATP and not UV irradiated. (B) Autoradiogram of the same gel. Arrows indicate PhoH.

into the pT7-7 expression vector to make pT7-7H. pT7-7H was introduced into *E. coli* BL21 (DE3), and synthesis of the PhoH protein was induced by IPTG. The overproduced 39-kDa PhoH protein was found exclusively in the insoluble fraction (data not shown). PhoH recovered from the insoluble fraction was solubilized with 6 M guanidine-HCl.

To prove that the 39-kDa protein corresponded to the *phoH* gene product, the band was eluted and its N terminus was determined. This sequence, M-V-T-S-X-T-G-H-V-L-D-N-Q-R-A, agreed with the predicted sequence except for the fifth residue Cys, which was not identified. We concluded that the 39-kDa protein was indeed the product of the *phoH* gene.

Photoaffinity labeling of PhoH with [γ-^{32}P]8N_3ATP. Since PhoH has consensus motifs aligned with various nucleotide-binding proteins (Fig. 4), we examined whether PhoH had an ATP-binding activity by photoaffinity labeling with the ATP analog [γ-^{32}P]8N_3ATP.

Figure 9 shows a Coomassie-stained gel of [γ-^{32}P]8N_3 ATP-treated proteins (panel A) and an autoradiogram of the same gel (panel B). The 39-kDa band was PhoH protein (Fig. 9A, lanes 2 and 3). One strong radioactive band corresponded to the PhoH position in the sample treated with the photoaffinity label (Fig. 9B, lane 2), while control samples did not show this band (Fig. 9B, lanes 1 and 3). We concluded that PhoH has an ATP-binding activity.

DISCUSSION

The *phoH* gene belonging to the phosphate regulon was identified and characterized. Expression of the *phoH* gene was controlled by two promoters. The P1 promoter was induced by phosphate limitation and dependent on PhoB, while the P2 promoter was constitutive with respect to phosphate concentration in the medium (Fig. 6).

Wanner et al. reported 55 independent Mu d1 mutants that showed a phosphate starvation-inducible (*psi*) Lac$^+$ phenotype (40). The sequences of *psi*::*lacZ*(Mu d1) chromosomal junctions of 44 such *psi* fusions were determined (27, 32a). We found that the *phoH* sequence contained the same sequences with the junction sequences of *psiH45*::*lacZ*(Mu d1), *psiH14*::*lacZ*(Mu d1), and *psiH16*::*lacZ*(Mu d1) (Fig. 3).

The *psiH* gene has been reported to be induced by phosphate, carbon, and nitrogen starvation and by UV irradiation (39). We examined the induction of *phoH* by carbon starvation or UV irradiation with ANCK10 strains carrying pKC244, pKC94, or pKC28 which contained operon fusions with P1 and P2, P1, or P2 promoters, respectively. We also examined induction with the chromosomal operon fusions in strains SE5008 (*phoH*::λ*plac*Mu53) and CSH66Δ*lac* (*phoH*::λ*plac*Mu53) under conditions of phosphate, carbon, or nitrogen starvation (39). Both the CAT assay of in vitro operon fusions and the β-Gal assay of in vivo operon fusions indicated that the P1 promoter was induced only by phosphate starvation and that the P2 promoter was constitutive and was not affected by these conditions (data not shown). We do not know why the different results on regulation were obtained by the two laboratories, but it may be attributed to the strain backgrounds.

The results of photoaffinity labeling of PhoH with [γ-^{32}P] 8N_3ATP as well as the sequence homology indicated that PhoH contains nucleotide-binding motifs. Since many proteins that contain the nucleotide-binding motifs also have ATPase activity (14), PhoH may have ATPase activity. We were unable to detect ATPase activity in our PhoH preparation, possibly because the protein was denatured.

The hydropathicity plot of PhoH, according to Kyte and Doolittle (19), indicates that the protein has a rather hydrophilic character, with a mean index of +0.11. Since hydrophobic-hydrophilic domains with spatial phase transitions

are not apparent in the protein, PhoH is probably a cytoplasmic protein. Since all *pho* genes are related to the transport and use of various forms of combined or free phosphate (31, 36, 38), the product of the *phoH* gene may also be involved in such functions.

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