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NOTE

Identification of PhoB Binding Sites of the *yibD* and *ytfK* Promoter Regions in *Escherichia coli*

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By using a *lacZ* operon fusion genomic library of the *Escherichia coli* O157:H7 Sakai, we identified phosphate-starvation-inducible (*psi*) promoters located upstream of the *yibD* and *ytfK* genes. They have been previously proposed to belong to the phosphate regulon (*pho* regulon) by Beak and Lee (2006), based on the DNA array and *in vivo* transcriptional experiments. However, the direct interaction of these promoters with the activator protein of the *pho* regulon, PhoB, has not been determined. We determined the binding regions of PhoB in these promoter regions by DNase I footprinting. Both regions contained two *pho* boxes similar to the consensus sequence for PhoB binding.

Keywords*: *Escherichia coli*, *pho* regulon, two-component regulatory system, *yibD*, *ytfK

In response to environmental phosphate limitation, *Escherichia coli* activates transcription of a series of genes whose products have roles in the transport and assimilation of various phosphorus compounds. These genes are under the transcriptional control of the PhoB and PhoR proteins which belong to a large family of two-component regulatory systems that respond to a variety of environmental stimuli (for review, see Wanner, 1996; Makino *et al*., 1998; Hsieh and Wanner, 2010). When the cells are subjected to phosphate starvation stress, the PhoR undergoes autophosphorylation with ATP and acts as a protein kinase for the transcriptional activator PhoB (Makino *et al*., 1992). Consequently, phosphorylated PhoB binds to the *pho* box, a consensus sequence of the *pho* regulon promoters, and activates transcription with RNA polymerase containing the major σ-factor σ^{70} (Makino *et al*., 1993, 1996; Kim *et al*., 1995; Okamura *et al*., 2007). In *E. coli* K-12 strains, at least eight operons (*phoA*, *phoB-R*, *phoE*, *phoH*, *psiE*, *pstS-phoU*, *phnC-P*, and *ugpB-Q*) consisting of 31 genes are known to be members of the *pho* regulon (Kikuchi *et al*., 1981; Wanner *et al*., 1981; Wanner and McSharry, 1982; Overbeeke *et al*., 1983; Amemura *et al*., 1985; Makino *et al*., 1986a, 1986b, 1991; Overduin *et al*., 1988; Kimura *et al*., 1989; Kasahara *et al*., 1991; Kim *et al*., 1993, 2000). Recent study have shown that the *amn*, *yibD*, and *ytfK* genes are activated by PhoB and these are proposed for the novel members of the *pho* regulon (Beak and Lee, 2006), though evidence for direct contact of PhoB to these promoter regions is lacking.

By using a *lacZ* operon fusion genomic library of the *E. coli* O157:H7 Sakai, we have identified a PhoB regulated promoter located upstream of the *ecs0540-ecs0544* genes that mapped within one of the strain-specific chromosomal regions of the *E. coli* O157:H7 and demonstrated that these genes belong to the *pho* regulon (Yoshida *et al*., 2010). In the process for identifying the PhoB regulated promoter, we also cloned the regulatory regions of the *yibD* and *ytfK*. Genomic DNA of the *E. coli* O157:H7 Sakai was shotgun-cloned into the low-copy number vector pMW222 (Yokoyama *et al*., 2005) carrying a promoter-less *lacZ* reporter gene to generate pMW222-library. These plasmids were introduced into the *lacZ*-deletion strain MC4100 (Silhavy *et al*., 1984) by electroporation, and a set of the transformants were selected on tryptone agar plates (Miller, 1992) containing 100 μg/ml of kanamycin. These colonies were then replicated by using filter papers (Whatman) onto TGLP (low phosphate) and TGHP (high phosphate) (Amemura *et al*., 1982) agar plates containing kanamycin and 40 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), a chromogenic substrate of β-galactosidase (β-gal). Transformants showing high activity of β-gal (dark blue) on TGLP and low activity (white or pale blue) on TGHP were isolated as clones carrying a pMW222 derivative containing a *psi* promoter. Each of the inserts of pMW222 derivatives isolated from these transformants was sequenced using the M13-47 primer (5′-CGCCAGGGTTTTCCCAGTC ACGAC-3′) and its position on the Sakai chromosome was identified as described (Oyamada *et al*., 2007; Yoshida *et al*., 2008, 2010; Sugiyama *et al*., 2010). Of these pMW222 derivatives, pMW222D and pMW222Y carried the 5′-flanking regions of the *yibD* and *ytfK*, respectively (Fig. 1), both of which were located within the "backbone region" (Hayashi *et al*., 2001) conserved among most *E. coli* strains including K-12 derivatives.

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Table 1. β-Galactosidase activities of the cells carrying pMW222 derivatives

Plasmid	β-Gal activity (SD)[a]			
	MC4100 (wild-type)		KM2000 [Δ(phoB-phoR)]	
	TGLP	TGHP	TGLP	TGHP
pMW222D	20235 (465)	38 (3)	65 (2)	54 (1)
pMW222Y	9522 (89)	838 (164)	1323 (50)	1203 (97)

[a] Overnight tryptone-broth cultures of the MC4100 (wild-type) and KM2000 [Δ(*phoB-phoR*)] strains carrying the indicated plasmids were diluted 100-fold into TGLP and TGHP media and the cells were grown aerobically at 37°C for 12 h to stationary phase. β-Gal activities were assayed as described by Miller (1992) in triplicate. The averages and standard deviations are expressed in Miller units.

To reveal whether high expression of β-gal under phosphate limitation from these *psi* promoters was controlled by the *phoB-phoR*, we examined the β-gal activities of the MC4100 (wild-type) and the Δ(*phoB-phoR*) derivative KM2000 (Yoshida *et al*., 2010) strains containing the pMW222D and pMW222Y. For this purpose, the pMW222D and pMW222Y were introduced into MC4100 and KM2000 strains and the transformants were selected on tryptone-agar plates containing kanamycin. Overnight cultures of these transformants were diluted 100-fold into TGLP and TGHP media and the cells were grown aerobically at 37°C for 12 h to stationary phase. β-Gal activities were assayed as described by Miller (1992). The β-gal activities of MC4100 cells carrying pMW222D and pMW222Y in TGLP were 533 and 11-fold higher than those in TGHP, respectively, whereas the β-gal activities of KM2000 cells carrying these plasmids in TGLP were approximately equal to those in TGHP, respectively (Table 1), indicating that transcriptional activations from the promoters within the fragments D and Y were dependent on the *phoB-phoR* under conditions of phosphate starvation. These data are in agreement with the previously described data by Beak and Lee (2006).

To define the functional promoter regions, the fragments of D and Y (see Fig. 1) were further shortened and cloned into pMW222 as follows. The D and Y fragments amplified by PCR from the pMW222D and pMW222Y using the LF3 (5′-GTGCTGCAAGGCGATTAAGTTG-3′) and 551up (5′-A CTGCCAGGAATTGGGGATCGG-3′) primers were sonicated, blunt-ended with the DNA blunting kit (TaKaRa, Japan), and separated by polyacrylamide gel electrophoresis. DNA fragments of 100-300 bp were extracted from the gel using MG elution buffer [500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% (w/v) SDS] and purified by ethanol precipitation, and ligated into the *Swa*I site of the pMW222 pretreated with *Swa*I and bacterial alkaline phosphatase (TaKaRa). These plasmids were introduced into MC4100 and transformants carrying a pMW222 derivative containing the *psi* promoter were selected as described above. pMW222D-7 and pMW222Y-1 carrying the D-7 and Y-1 fragments (Fig. 1), respectively, were selected.

Next, by using the fragments D-7 and Y-1, we performed DNase I footprinting experiment to examine whether the PhoB protein directly binds to these *psi* promoters. The *phoB* gene fragment amplified by PCR using primers, *phoB-Nde*I (5′-CCCCATATGGCGAGACGTATTCTGGTCG-3′) and *phoB-Hin*dIII (5′-CCGAAGCTTAAAAGCGGGTTGAAAAACG-3′), was digested with *Nde*I and *Hin*dIII restriction enzymes, and cloned into the corresponding sites of the plasmid pET28a(+) (Novagen) containing an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 promoter to construct a His-tagged PhoB overproduction plasmid, pET28a(+)-*phoB*. This plasmid was introduced into *E. coli* BL21 (λDE3) (Studier and Moffatt, 1986), and His-tagged PhoB protein was overproduced by adding 1 mM of IPTG to the medium and purified by Ni-NTA Agarose (QIAGEN) in accordance with the protocol (QIA expressionist). Overproduction and purification of PhoR1084

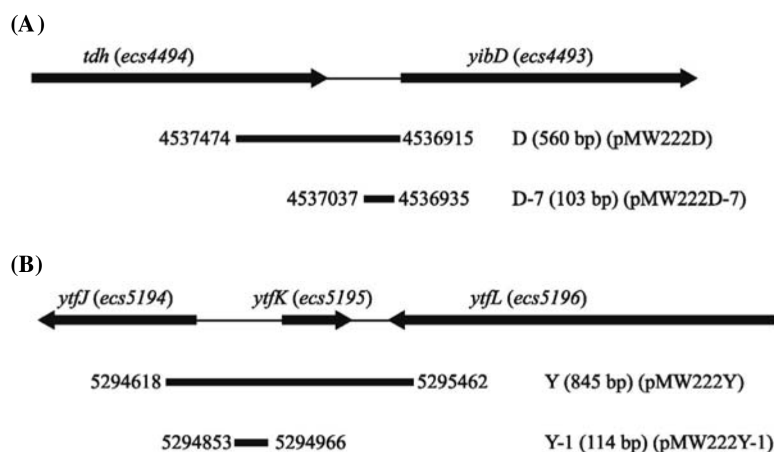

Fig. 1. Schematic presentation of the *yibD* (A) and *ytfK* (B) regions. Horizontal bars indicate DNA fragments cloned into pMW222. The seven-digit numbers indicate the chromosomal positions in the genome database of *E. coli* O157:H7 Sakai (http://genome.bio.titech.ac.jp/bacteria/o157/index.html). The lengths of the fragments and the plasmid names are given in parenthesis, respectively.

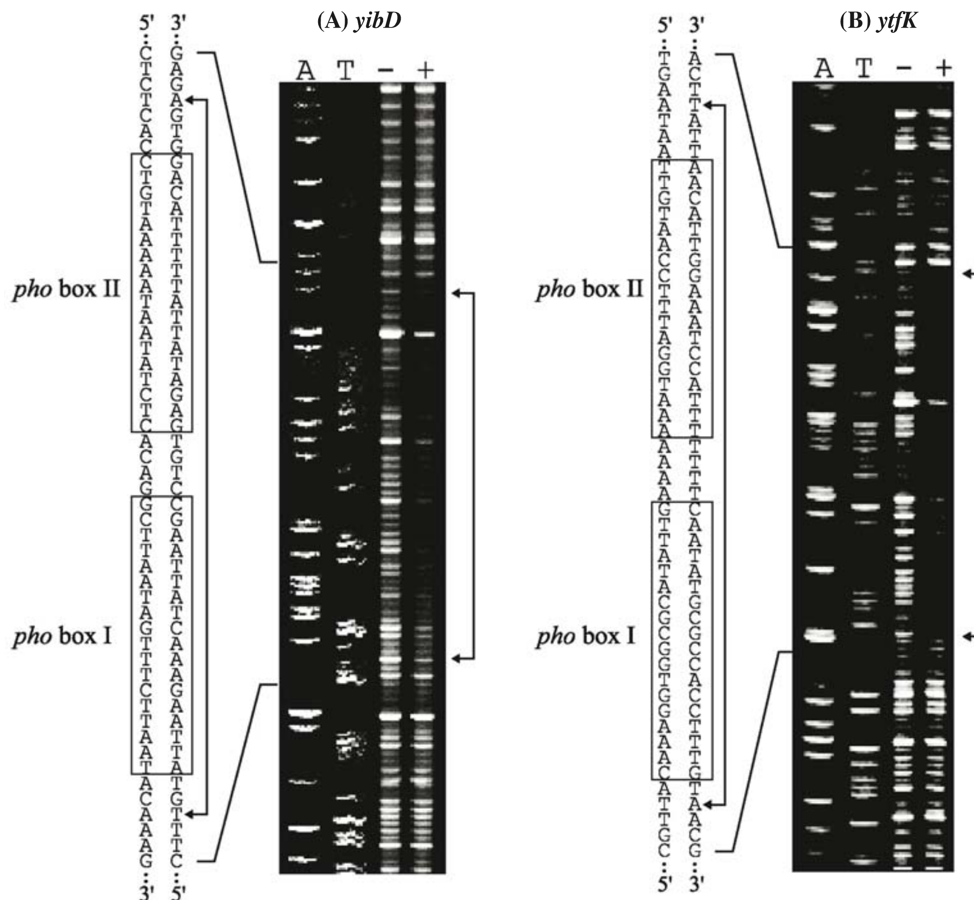

Fig. 2. DNase I footprinting of the *yibD* (A) and *ytfK* (B) promoters. The 5′-labeled DNA probes of the D-7 and Y-1 fragments (Fig. 1) were digested by DNase I in the absence (lane -) or presence (lane +) of both PhoR1084 and PhoB proteins as described in the text. Lanes A and T represent the dideoxy sequencing ladders derived from the same fragments. The brackets with arrows show regions protected from DNase I digestion in the presence of PhoR1084 and PhoB. The sequences similar to the consensus sequence of PhoB binding are boxed with *pho* box I and II, respectively.

protein, a truncated form of PhoR protein lacking the amino-terminal hydrophobic region, was described previously (Makino *et al*., 1989). The protein is locked in a form to activate PhoB, that is to say, it constitutively activates PhoB by phosphorylation, irrespective of phosphate levels of the medium *in vivo*. The fluorescently labeled DNA probes of the D-7 and Y-1 fragments, which were prepared by PCR amplification from the pMW222D-7 and pMW222Y-1, respectively, using the LF3 primer and the -21M13 primer (5′-GTGCTGCAAGGCG ATTAAGTTGG-3′) labeled with 6-FAM at the 5′-end, described

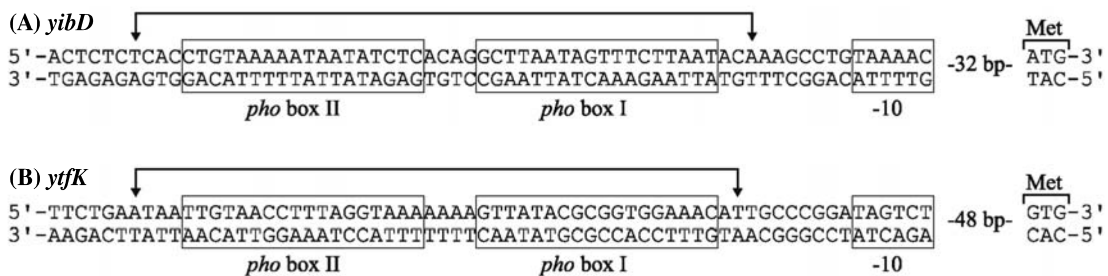

Fig. 3. Promoter sequences of the *yibD* (A) and *ytfK* (B). The *pho* box sequences and the putative -10 sequences are boxed. The start codons and the numbers of the nucleotides from the -10 sequences are indicated. The brackets with arrows correspond regions protected from DNase I digestion in the presence of PhoR1084 and PhoB.

Table 2. Comparison of the *pho* box sequences

Gene	*pho* box[a]
phoA	CTGTCATaaagTTGTCAC
phoB	TTTTCATaaatCTGTCAT
phoE	CTGTAATatatCTTTAAC
phoH	CTGTCATcactCTGTCAT
phnC	CTGTTAGtcacTTTTAAT
psiE I	AATATAGatctCCGTCAC
psiE II	GTTGAACaaaaCATACAC
pstS I	CTTACATataaCTGTCAC
pstS II	CTGTCATaaaaCTGTCAT
ugpB I	AAGTTATttttCTGTAAT
ugpB II	CTATCTTacaaATGTAAC
ugpB III	TTGTCATctttCTGACAC
ecs0540	CTTTAATaaatAAGTCAC
yibD I	GCTTAATagttTCTTAAT
yibD II	CTGTAAAaataATATCTC
ytfK I	GTTATACgcggTGGAAAC
ytfK II	TTGTAACctttAGGTAAA
Consensus	CTGTCATaaatCTGTCAC

[a] Previously identified *pho* boxes are derived from Kim *et al*. (2000) (*psiE*), Yoshida *et al*. (2010) (*ecs0540*), and Makino *et al*. (1998) (the others).

as previously (Oyamada *et al*., 2007; Yoshida *et al*., 2010), were mixed with PhoR1084 in a total volume of 100 μl of footprint buffer (20 mM Tris-HCl; pH 7.9, 3 mM $MgCl_2$, 5 mM $CaCl_2$, 0.1 M NaCl, 0.1 mM EDTA, 0.1 mM DTT, 1 mM ATP, and 50 μg/ml bovine serum albumin) and incubated at 37°C for 10 min. Then, PhoB was added and the mixtures were incubated at 30°C for 5 min. After 20 ng of DNase I was added, the mixtures were incubated at 30°C for 1 min. The reactions were stopped by adding 50 μl of TE-saturated phenol and 25 μl of stop solution (1.5 M sodium acetate; pH 5.2, 20 mM EDTA, and 100 μg/ml tRNA). The reaction products were purified by ethanol precipitation and detected by ABI PRIZM 377 DNA sequencer as described (Oyamada *et al*., 2007; Yoshida *et al*., 2010). The corresponding A and T sequencing ladders were made by using *fmol* DNA Cycle Sequencing System (Promega). Protections by PhoB from DNase I digestion were observed in the regions spanning nucleotides -92 to -46 and -108 to -63 upstream of the start codons of the *yibD* and *ytfK*, respectively (Figs. 2 and 3). These regions included two sets of *pho* box sequences that are, to some extent, similar to the consensus sequence (Table 2). These results indicate that expression of the *yibD* and *ytkK* genes is directly regulated by PhoB and therefore these genes are involved in the *pho* regulon.

All of previously identified *pho* regulon promoters have putative -10 promoter sequences 10 bp downstream from the *pho* box sequences and do not have the sequences similar to the -35 promoter sequences (Makino *et al*., 1998; Yoshida *et al*., 2010). In agreement with these, we found the putative -10 sequences similar to the consensus (5′-TATAAT-3′) and did not find the putative -35 sequences similar to the consensus (5′-TTGACA-3′) within these promoter regions (Fig. 3). Among the previously identified *pho* regulon promoters, the *pst*, *ugp*, and *psiE* promoters contain more than one *pho* box sequence (Makino *et al*., 1988; Kasahara *et al*., 1991; Kim *et al*., 2000), while the other *pho* regulon promoters contain only one unit of the box. Our results have revealed that both the *yibD* and *ytfK* promoter regions contain two units of *pho* boxes and hence these genes belong to the *pho* regulon with the former classification. The PhoB binding sites experimentally determined here were quite different from these previously predicted by the computer sequence analysis by Beak and Lee (2006). In some cases, it is difficult to predict PhoB binding sequences because, as shown in Table 2, some *pho* boxes are not similar to the consensus *pho* box so much. Great caution should be exercised when identifying binding sites based only on sequence predictions.

Based on sequence similarity, the *yibD* gene is predicted to encode a glycosyltransferase (Serres *et al*., 2001), a ribulose-bisphosphate carboxylase, and/or an inosine triphosphate diphosphatase (Reed *et al*., 2003), whereas the function of the *ytfK* gene is unknown. Inosine triphosphate diphosphatase catalyzes dephosphorylation of inosine triphosphate. These observations suggest that the product of the *yibD* gene is related to phosphate metabolism. The functions of these genes will be revealed soon.

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