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ORIGINAL ARTICLE

Functional Identification of OphR, an IclR Family Transcriptional Regulator Involved in the Regulation of the Phthalate Catabolic Operon in *Rhodococcus* sp. Strain DK17

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Abstract A putative gene for a transcriptional regulator (*ophR*) was detected near each copy of the duplicated phthalate-degrading operon of *Rhodococcus* sp. DK17. Sequence analysis and molecular modeling indicate that OphR belongs to the IclR family of transcriptional regulators and possesses the N-terminal DNA-binding and C-terminal effector-binding domains. DNA-binding assays demonstrate that OphR regulates the phthalate operon by binding to the *ophA1-ophR* intergenic region.

Keywords Phthalate · Regulator · *Rhodococcus* sp. strain DK17 · Transcription initiation

Introduction

Phthalate (1,2-dicarboxybenzene) is a key metabolic intermediate in the aerobic degradation of phthalate esters and of some polycyclic aromatic hydrocarbons including pyrene and phenanthrene [1]. In general, Gram-positive bacteria initially oxidize phthalate to 3,4-dihydro-3,4-dihydroxyphthalate, which is subsequently dehydrogenated and decarboxylated to form protocatechuate [2–4]. On the other hand, phthalate degradation in Gram-negative bacteria proceeds through oxygenation and dehydrogenation at carbons 4 and 5 to form 4,5-dihydroxyphthalate, followed by decarboxylation to yield protocatechuate [5].

Alkylbenzene-degrading *Rhodococcus* sp. strain DK17 is also able to utilize phthalate as a growth substrate via the Gram-positive pathway [6, 7]. DK17 possesses three megaplasmids (380 kb pDK1, 330 kb pDK2, and 750 kb pDK3), and the phthalate operons in DK17 are duplicated and are present on both pDK2 and pDK3 [6, 8]. It was previously shown that both copies of the phthalate operon are equally functional in DK17 [9]. Each phthalate operon contains the genes *ophA1-ophA2-orf0-ophB-ophA3-ophA4-ophC*, encoding the large and small subunits of a terminal oxygenase component of phthalate 3,4-dioxygenase, a protein of unknown function, a phthalate dihydrodiol dehydrogenase, a ferredoxin component of phthalate 3,4-dioxygenase, a ferredoxin reductase component of phthalate 3,4-dioxygenase, and a decarboxylase, respectively (Fig. 1). A putative regulatory gene, *ophR*, is located upstream of each operon.

One interesting aspect of phthalate degradation by DK17 is that benzoate completely inhibits the expression of the phthalate operon in DK17 through a catabolite repression-like mechanism [10]. That finding provided new insights into the bacterial degradation of aromatic hydrocarbons within a mixture of analogous compounds, although more extensive studies are needed to completely understand the catabolite repression-like response of DK17. The present work was initiated as a first step towards elucidating the regulatory mechanisms underlying phthalate metabolism by DK17.

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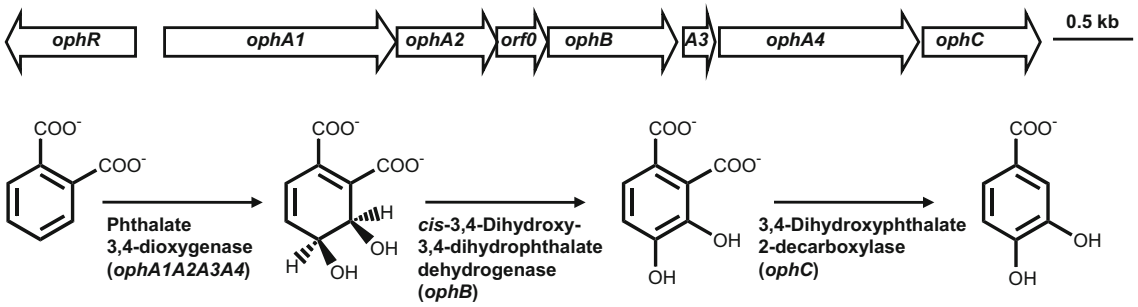

Fig. 1 Pathway for the initial degradation of phthalate with the phthalate operon in *Rhodococcus* sp. strain DK17

Materials and Methods

Preparation of Cell Extracts and N-Terminal Amino Acid Sequencing of OphA1

Rhodococcus sp. strains DK176 and KC810 were derived from DK17 by curing pDK2 [11] and pDK3 [7], respectively. Initially, DK176 and KC810 were grown overnight in 50 ml mineral salts basal (MSB) medium containing 20 mM glucose. The cells were then harvested, washed, and resuspended in 100 ml fresh MSB medium containing 5 mM phthalate at an initial optical density at 600 nm (OD_{600}) of approximately 0.1 and then further incubated at 30 °C for 12 h with shaking (180 rpm).

The induced cells were harvested, washed in a half volume of 1× (phosphate-buffered saline [PBS], 140 mM NaCl, 2.7 mM KCl, 10 mM $NaHPO_4$, and 1.8 mM KH_2PO_4[pH 7.4]), suspended in 5 ml 50 mM 3-morpholinopropanesulfonic acid buffer (pH 7.8) containing 1 mM ascorbic acid, 10 % acetone, 10 % glycerol, and 100 μM $FeSO_4$, and then disrupted by sonication. The unbroken cells and cell debris were removed by centrifugation at 10,000×*g* for 30 min, and the clear supernatant solutions were further centrifuged at 100,000×*g* for 90 min. The concentrations of the resulting protein solutions were determined using Coomassie Protein Assay Reagent (Pierce, USA) with bovine serum albumin (BSA) as the standard. SDS-PAGE was performed on 12.5 % acrylamide gel in Hoefer Mighty Small SE245 electrophoresis cells (Amersham Biosciences, UK). The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The appropriate Coomassie-stained protein band was excised from the PVDF membrane and installed into the blot cartridge of a ProcisecLC 492 protein sequencer (Applied Biosystems, USA) for N-terminal sequence analysis.

Heterologous Expression and Purification of OphR

ophR was PCR amplified and cloned into the *Escherichia coli* expression vector pEXP5-CT/TOPO to construct the recombination plasmid. *E. coli* BL21(DE3)-*ophR* was grown at 37 °C in LB broth until the OD_{600} reached approximately 0.5–0.7, and then IPTG was added to a final concentration of 1 mM to express the His-tagged OphR protein. The cells were harvested after 2 h and resuspended in binding buffer (20 mM Tris pH 7.9, 0.2 M NaCl, and 5 mM imidazole). The cells were then lysed using BugBuster Master Mix (Novagen), and the cell debris was removed by centrifugation. The soluble material was loaded onto a Ni^{2+} metal chelation column (Amersham Biosciences) equilibrated with binding buffer. After extensive washing, the bound protein was eluted with elution buffer [20 mM Tris (pH 7.9), 0.2 M NaCl, and 200 mM imidazole].

Primer Extension Analysis

In order to determine the transcriptional initiation site of each phthalate operon, a primer extension experiment was performed with total RNA extracted from phthalate-induced DK176 and KC810 cells, respectively. A total RNA extraction was then carried out as described previously [10]. Each total RNA sample (40 ng) was reverse transcribed in 20 μl reaction mixture at 55 °C for 30 min with Superscript Reverse transcriptase (Invitrogen, USA) and a reverse primer (*ophA1*-PE-F: 5′-GTCGTCCGGGGTTCC AAGGTTC-3′) complementary to the 5′-end sequence of the *ophA1* genes. The primer extension products were purified by precipitation in ethanol and dissolved in 8 μl loading dye solution [95 % (v/v) formamide, 20 mM EDTA, 0.05 % (w/v) bromophenol blue]. After the samples were heated at 90 °C for 2 min, they were separated on an 8 % polyacrylamide sequencing gel containing 8 M urea. For the separation, DNA sequence ladders were prepared using the same primer and with pGEMT-Easy-*ophA1-ophR* as a template according to the instructions provided with the Thermo Sequenase Cycle Sequencing kit (USB, USA).

OphR-DNA Binding Assays

Binding reactions were performed with approximately 10 fmol labeled 156 bp *ophR-ophA1* intergenic region (IR)

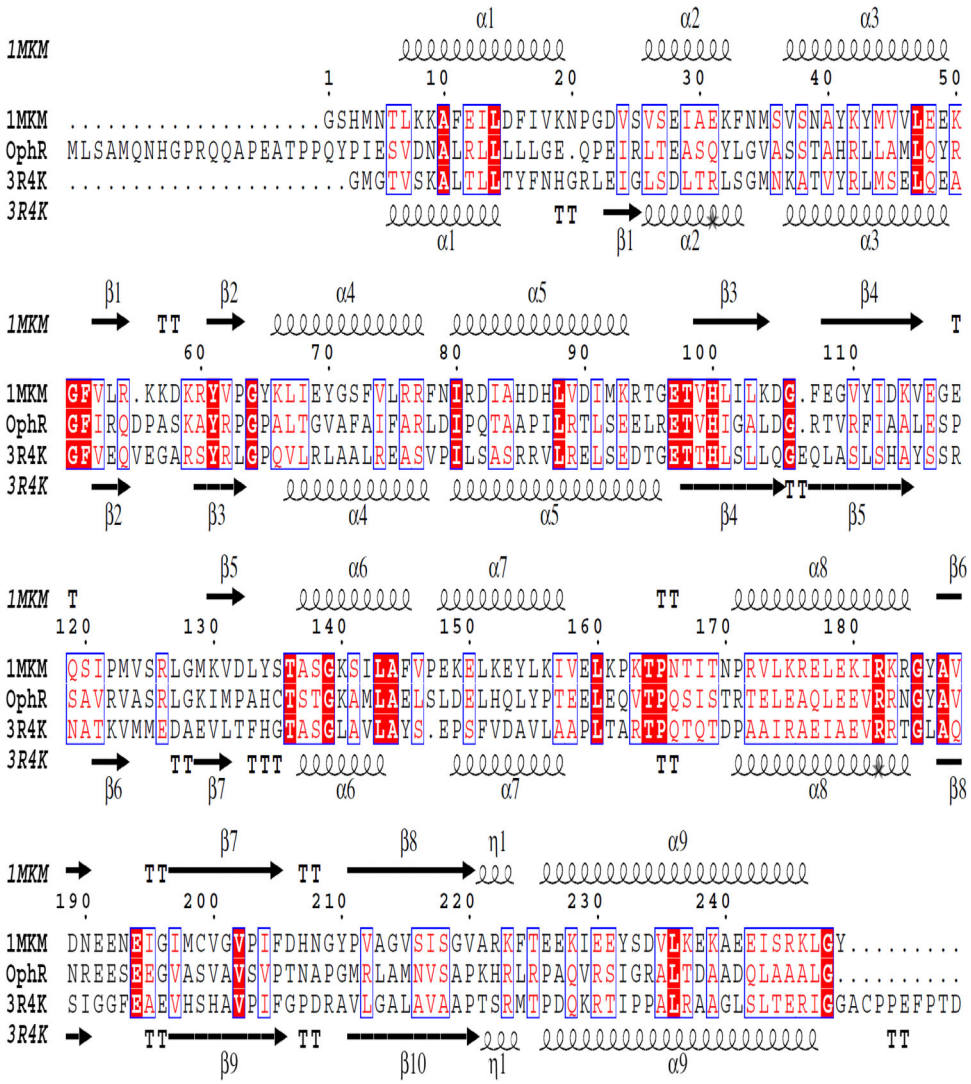

Fig. 2 Sequence alignment of OphR from *Rhodococcus* sp. strain DK17 with IclR transcriptional factors. The amino acid sequence of OphR was aligned with those of two IclR transcriptional factors with crystal structures; PDB Ids: 1MKM (*T. maritima* IclR) and 3R4 K (*Silicibacter* sp. TM1040 IclR). Elements of the secondary structures of the IclR transcriptional factors from *T. maritima* and *Silicibacter* sp. TM1040 are shown above and below the alignments, respectively. The sequences were aligned using ClustalW2 and visualized using the ESPript software

DNA and 6.8–6,800 fmol purified OphR in 20 µl binding buffer (100 mM HEPES [pH 8.0], 100 mM KCl, 5 mM EDTA, 5 mM DTT, 30 mM $MgCl_2$, 40 % glycerol, and 0.1 µg poly[dI-dC]). Following incubation at room temperature for 30 min, the binding mixture was subjected to electrophoresis at 25 °C on a 5 % polyacrylamide gel at 200 V in 1× TBE buffer. After electrophoresis, the gel was dried and exposed to a phosphorimage screen for viewing with a phosphorimage analyzer (FLA-2000, Fuji).

Results and Discussion

In Silico Analysis of the Putative Regulatory Protein OphR

A sequence alignment of the pDK2-encoded and pDK3-encoded OphR proteins revealed four amino acid substitutions among 268 amino acid residues: T42P, T77A, V150I, and K204N. A Blastp analysis showed that the DK17 OphR proteins match well with the putative IclR family transcriptional regulators from members of the *Rhodococcus* and *Mycobacterium* genera and possess a potential IclR helix-turn-helix domain for binding. Subsequently, computer-aided molecular modeling studies were performed to gain more insight into the functional characteristics of the OphR proteins.

The deduced amino acid sequence of the pDK3-encoded OphR was aligned with the IclR transcriptional factors from *Thermotoga maritima* TM0065 (PDB: 1MKM) and *Silicibacter* sp. TM1040 (PDB 3R4 K), because those were the most similar sequences among the IclR transcription factors for which crystal structures were available. As shown in Fig. 2, OphR has the N-terminal DNA-binding domain and the C-terminal effector-binding domain, which are general properties of the IclR family proteins, and it has

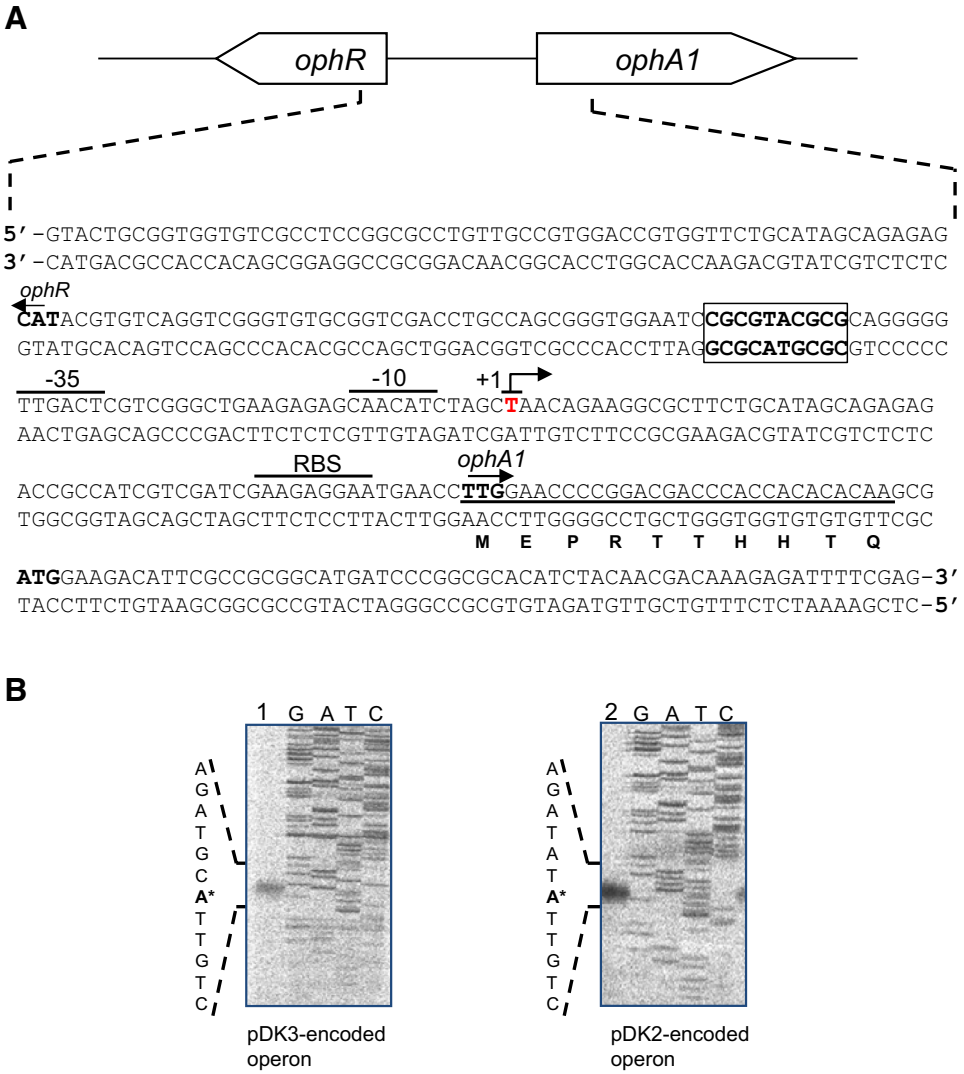

Fig. 3 Dissection of the *ophA1-ophR* intergenic region. **a** A representation of the intergenic region between *ophA1* and *ophR*. The *ophA1* and *ophR* genes are represented as large *white arrows*, respectively. The putative ribosome-binding site is labeled as RBS (Shine-Dalgarno sequence). The *bold red* nucleotide T with a *broken arrow* indicates the transcription start site of *ophA1*, as determined by the primer extension analyses. The −35 and −10 sequences of the putative σ70-type promoters for each gene are denoted. The putative OphR-binding site is labeled with a *black box*. **b** Mapping of the transcription start site for *ophA1* and *ophA1′*. Primer extension reactions were performed with pDK3-encoded total RNAs isolated from phthalate-induced DK176 cells and with pDK2-encoded RNAs isolated from phthalate-induced KC810 cells. The extended products (*lanes 1* and *2*) were analyzed with sequencing ladders generated using the same primer and template (*lanes G*, *A*, *T*, and *C*). The nucleotide sequence deduced from the sequencing lanes is shown on the *left*. The first nucleotide of the most intense transcript for each gene is denoted by an *asterisk*

additional residues at its N-terminus compared with the other two IclR transcriptional factors. Helices α2 and α3 form a helix-loop-helix (HLH) motif, which is the DNA-binding motif (for DNA sequence recognition). Although the sequence in that region is specific for the DNA recognition sequence, conserved residues (D/E in helix α2 and M/L in helix α3 following G) [12] are also found in OphR. In particular, it is noteworthy that the amino acid substitutions observed between the pDK2-encoded and the pDK3-encoded OphR proteins are located at the positions corresponding to the loops connecting helices α1 and α2, β1 and β2, the β5 strand, and the end of helix α8 in the *T. maritima* IclR. It is therefore unlikely that those amino acid substitutions affect the conformation and function of the protein.

Identification of the Regulatory Regions of the DK17 Phthalate Operons

As a first step to identify regulatory regions, the translational start site of each operon was identified by determining the N-terminal amino acid sequences of the pDK2-

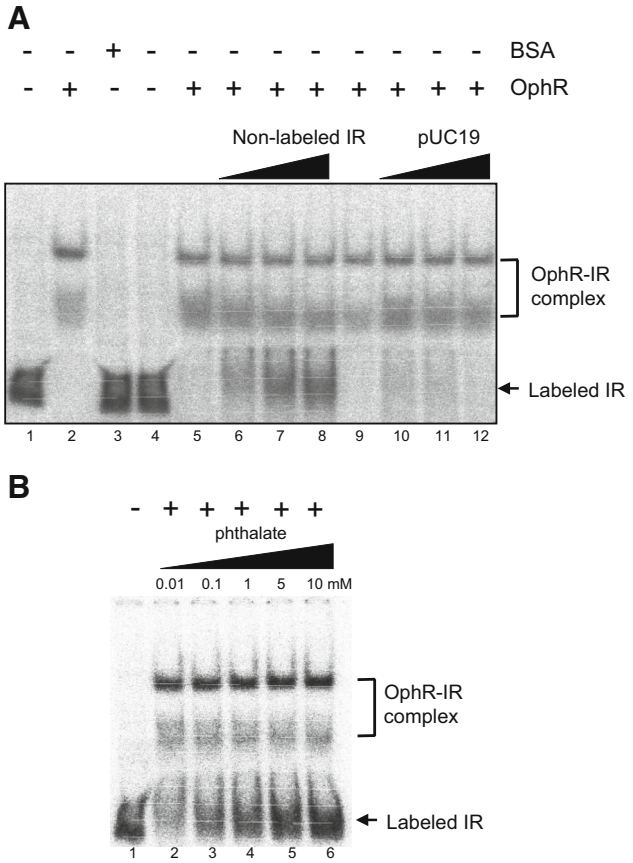

Fig. 4 OphR binding activity with the *ophA1-ophR* intergenic region. **a** The binding activity of OphR. A gel mobility-shift assay was performed with the labeled, 120 bp DNA fragment from the *ophA1-ophR* intergenic region (IR). *Lane 1* is a control lane containing 10 nM labeled DNA fragment only. *Lanes 2* and *3* contain the DNA probe incubated with 10 nM OphR and 10 nM BSA, respectively. The positions of the free DNA and the OphR-IR complex are indicated. Competition assay: *lane 4* is a control lane containing 10 nM labeled DNA fragment only; *lanes 5* and *9* contain the DNA probe incubated with 10 nM OphR; *lanes 6–8* contain the DNA probe incubated with 10 nM OphR in the presence of 10, 20, and 40 nM non-labeled DNA of the same intergenic region, respectively; lanes 10–12 contain the DNA probe incubated with 10 nM OphR in the presence of 10, 20, and 40 nM competitive DNA derived from pUC19, respectively. **b** Modulatory effects of phthalate on the DNA-binding activity of OphR. Gel mobility-shift patterns obtained in the presence of phthalate. *Lane 1* is a control lane containing the labeled DNA fragment only; *lanes 2–6* contain the DNA probe incubated with 20 nM OphR in the presence of increasing amounts of phthalate from 10 μM to 10 mM. The positions of the free DNA and the OphR-DNA complex are indicated

encoded and pDK3-encoded OphA enzymes, respectively. As explained in the Introduction, the pDK2-encoded and pDK3-encoded phthalate operons are equally functional in DK17. Accordingly, two previously constructed mutant strains, the pDK2-cured DK176 [11] and the pDK3-cured KC810 [7], were grown on glucose and phthalate, respectively, and the total cellular protein patterns were compared. The soluble cell extracts were separated on one-dimensional SDS-PAGE (data not shown). Because the sizes of both conceptually translated OphA1 proteins were expected to be approximately 50 kDa, each protein band of that size that was specifically produced in the phthalate-induced DK176 and KC810 cells was selected for N-terminal amino acid sequencing. The N-terminal amino acid sequence of each protein was determined to be MEPRTTHHTQ, indicating that both *ophA1* genes begin with a rather unusual start codon (TTG), which is 33 bp upstream of the initially annotated ATG (Fig. 3a).

Next, in order to determine the transcriptional initiation site of each operon, primer extension experiments were performed with total RNA extracted from DK176 and KC810 cells, respectively, following induction with phthalate as described in the Materials and Methods. As shown Fig. 3b, both transcriptional start sites were identified as an A located 62 bp upstream from the respective *ophA1* translational start sites. A typical −35 region (TTGACT), which shows 100 % identity with the consensus sequence of the *E. coli* σ^{70}-recognized promoter, was found upstream of the transcriptional start site (Fig. 3a). A putative −10 region (CAACAT) was also detected 17 bp downstream of the −35 region. It should be noted that the putative promoter sequences of both *ophA1* genes are exactly identical to each other, although the whole intergenic region shares 96 % identity between *ophA1* and *ophR*.

Heterologous Expression, Purification, and DNA-Binding Activity of the OphR Derived From the pDK3 Plasmid

The molecular modeling results suggest that the two OphR proteins are functionally identical, because the differences between them are neither in the DNA-binding motif nor in the interfaces for subunit interaction. We therefore focused on the OphR protein encoded on pDK3 and examined its DNA-binding activity with the *ophR-ophA1* IR. The *ophR* gene was amplified, cloned, expressed, and purified as described in the Materials and Methods. The binding reactions were performed with approximately 10 fmol labeled 156 bp IR DNA and 6.8 to 6,800 fmol purified OphR in 20 μl binding buffer.

As shown in Fig. 4a, gel retardation assays demonstrated that OphR bound a radiolabeled probe containing the 156 bp region of the IR (lane 2), while BSA could not bind the same probe. More importantly, the binding of OphR to the probe decreased as increasing amounts of the non-labeled IR were added (lanes 6–8). In contrast, no detectable effects on the OphR binding were seen when pUC19 DNA was added as a competitor against the radiolabeled IR (lanes 10–12). Those observations indicate

that OphR specifically binds to the probe. Furthermore, the OphR-IR complex was dissociated by the addition of phthalate (Fig. 4b). It is generally known that the respective binding sites of RNA polymerase and the repressor overlap in most IclR-type regulators so that the repressor blocks the RNA polymerase binding [13, 14]. Combined with that fact, the dissociation of the OphR-IR complex by phthalate suggests that OphR acts as a repressor of the expression of the phthalate operon by occupying the regulatory region. Overall, the current work used a combination of sequence analysis, molecular modeling, and DNA-binding assays to show that OphR belongs to the IclR family of transcriptional regulators and regulates the phthalate operon by binding to the *ophA1*-ophR intergenic region. This in turn provides a solid basis for further research on how benzoate represses phthalate catabolism in DK17.

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