Novel Regulator MphX Represses Activation of Phenol Hydroxylase Genes Caused by a XylR/DmpR-Type Regulator MphR in *Acinetobacter calcoaceticus*

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**Abstract**

*Acinetobacter calcoaceticus* PHEA-2 utilizes phenol as its sole carbon and energy source and has a multi-component phenol hydroxylase-encoding gene operon (mphKLMNOP) for phenol degradation. Two additional genes, mphR and mphX, were found upstream and downstream of mphKLMNOP, respectively. The mphR gene encodes a XylR/DmpR-type regulator-like protein and is transcribed in the opposite direction to mphKLMNOP. The mphX gene is transcribed in the same direction as mphKLMNOP and encodes a protein with 293 amino acid residues showing weak identity with some unknown proteins encoded in the meta-cleavage pathway gene clusters for aromatic compound degradation. Disruption of mphR by homologous recombination resulted in the loss of phenol degradation while disruption of mphX caused significantly faster phenol degradation than in the wild type strain. Transcriptional assays for mphK, mphR, and mphX revealed that mphR activated mphKLMNOP transcription in the presence of phenol, but mphX partially repressed this activation. Gel mobility-shift assay demonstrated a direct interaction of MphR with the mph promoter region. These results indicate the involvement of a novel repressor protein MphX in transcriptional regulation of phenol hydroxylase genes caused by a XylR/DmpR-type regulator MphR.

**Introduction**

Besides being important industrially for the synthesis of chemical products such as resins, medicines, and dyes, phenol is also a major toxic contaminant in industrial wastewaters from oil refineries, petrochemical plants, and phenolic resin plants. Thus, efforts to improve the efficiency of wastewater treatment via phenol degradation have gained special attention. Such efforts have led to a deeper understanding of phenol biodegradation mechanisms [1,2].

A large variety of soil bacteria are able to degrade aromatic compounds by employing different strategies for regulatory control at the level of gene expression. In aerobic phenol degradation by microorganisms, phenol is normally converted by phenol hydroxylase into catechol, which flows into an ortho-cleavage pathway or a meta-cleavage pathway after the cleavage of an aromatic-ring by catechol 1,2-dioxygenase or catechol 2,3-dioxygenase [2]. Phenol hydroxylase, catalyzing the initial reaction in phenol degradation, is a key rate-limiting enzyme. Many phenol hydroxylases from soil microorganisms can be classified into three groups: single-[3,4], two- [5,6], and multi-[7,9,9] component types. Microorganisms with multi-component phenol hydroxylase (mPH) are distributed worldwide and seem to be the most ubiquitous in the environment [10]. The mechanism of phenol oxidation by mPH and the localization, organization, expression, and regulation of the mPH-encoding genes have been investigated in order to gain a basic understanding of the mechanisms involved in phenol biodegradation [6,7,11,12,13,14,15,16] and to develop practical applications, such as the treatment of soils contaminated with aromatic compound and the development of biosensors for the detection of such contaminants [13,17]. The best-characterized example of the mPH-mediated pathway is the dmp/Dmp system of *Pseudomonas* sp. CIp600 consisting of 15 *dmp* genes on the pV1150 plasmid [9,18], where the upstream activator DmpR in the presence of aromatic effectors activates divergent transcription of the mPH operon (*dmpKLMNOP*) [18,19]. This gene arrangement is highly conserved amongst several other phenol degradation gene clusters such as *nph*, *phe*, *aph*, *phi*, and *bph* [7,20,21,22,23]. DmpR and its analogous regulators including XylR for toluene and xylene degradation [24,25] form a large XylR/DmpR subclass in the NtrC protein family [26,27]. The molecular mechanisms of xyl/Xyl and *dmp*/Dmp systems are well-characterized to be via
activator binding to σ^70-dependent promoter regions of respective target operators [14,24,23]. In addition to XylR/DmpR-type mediated regulation, co-regulation of mPH gene expression by different types of regulators has also been observed in Comamonas testosteroni strains TA441 and R5 [7,23,28,29]. Furthermore, carbon catabolite repression and some host-dependent global regulations have been reported to affect the primary regulation by XylR/DmpR-type proteins [14,30,31].

In most cases, the catabolic pathway-specific regulators act as transcriptional activators of gene expression with few exceptions of GntR-type proteins, which are exclusively described as repressors. The use of repressors in the regulation of phenol degradation is not yet fully understood. We described as repressors. The use of repressors in the regulation of exceptions of GntR-type proteins, which are exclusively transcriptional activators of gene expression with few transcriptional units, mphpKLMNOP, benM, benABCDE, and benKP, in this order. The complete genome of A. calcoaceticus PHEA-2 was sequenced recently and has been deposited in GenBank under accession number CP002177. Establishment of the complete genome sequence enabled mapping of the entire catabolic gene cluster in the PHEA-2 chromosome. In this study, we compared organization of the phenol catabolic gene cluster in A. calcoaceticus PHEA-2 (mph) and with the other phenol degrading genes cluster. In addition, the result of the growth test on phenol and measurements of phenol-oxygenating activities suggested that phenol degradation in PHEA-2 was inducible and that mphR and mphX encoded an activator and a repressor, respectively, affecting expression of the mPH-encoding genes.

Results

Involvement of the mph genes in phenol degradation

As described in the introduction, two gene candidates, mphR and mphX, were found upstream and downstream of the mph operon, respectively. From sequence analysis, mphpKLMNOP was found to encode mPH proteins, catalyzing the initial reaction in phenol degradation (Fig. 1A). This multi-component enzymes exhibits 38–72% and 58–93% identity with proteins encoded by the cognate genes mphpKLMNOP of Pseudomonas sp. CF600 [9] and mphpKLMNOP of A. calcoaceticus NCIB8250 [8], respectively. A mphR-deletion mutant lacking the center portion of the mPH-operon (Fig. 1B) was constructed by homologous recombination and named A2R. It lost its phenol degradation activity (Fig. 2), but was still able to assimilate catechol (data not shown), showing that mphpKLMNOP is essential for the initial step of phenol degradation in PHEA-2.

The MphR protein showed significant identity with XylR/DmpR-type regulators MphR (70% aa sequence identity), PhlR (52% identity), DmpR (51% identity), PhlR (51% identity), CapR (51% identity) and XylR (48% identity), and expected to encode a XylR/DmpR-type regulatory protein homolog (Fig. 3A). To clarify the function of mphR in phenol degradation, a mphR-deletion mutant was constructed from PHEA-2 by homologous recombination and named A2R. The deletion was confirmed by PCR (the expected deleted segment is shown in Fig. 1B). As shown in Fig. 2, A2R was unable to grow on or degrade phenol at all. Introduction of the wild-type mphR into A2R using a complementation plasmid resulted in recovery of phenol degradation activity (data not shown). These results indicate that mphR encodes an activator for the expression of mphpKLMNOP required for phenol degradation in PHEA-2.

In addition, we compared organization of the phenol catabolic gene cluster in A. calcoaceticus PHEA-2 (mph) and with the other phenol degrading genes cluster in A. calcoaceticus NCIB8250 (moph), C. testosteroni TA441 (mphp) and C. testosteroni R5 (phc) (Fig. 1B). We found that an 882-bp open reading frame named mphX was detected between mphpKLMNOP and benM and its gene product showed no significant similarity with any known functional proteins. To investigate the involvement of mphX in phenol degradation, an mphX-deletion mutant was constructed from PHEA-2 by homologous recombination and named A2X. Interestingly, mphX-deletion mutant A2X showed faster growth on phenol and faster phenol degradation than the wild-type PHEA-2 strain (Fig. 2). The mphX-complemented strain recovered the normal ability to grow on phenol with the growth rate similar to that of wild type (data not shown). In addition, in the phenol-oxygenating activity assay using cell suspensions, phenol-induced A2X cells showed higher activity (1.97±0.14 μmol/min/g-dry cell) compared to phenol-induced...
PHEA-2 cells (1.38±0.11 μmol/min/g-dry cell). However, lactate-grown cells of both strains showed similar basal level of activities (<0.05 μmol/min/g-dry cell). Therefore, it is possible that mphX encodes a repressor protein for the expression of the mph genes. The MphX protein is comprised of 293 aa residues and is expected to have a molecular mass of 32.2 kDa. It shows weak identity with proteins encoded by mphX (27.8% aa sequence identity) in the phenol degradation gene cluster of Comamonas testosteroni TA411 [34] and mphU (26.3% identity) in the aniline degradation gene cluster of Delftia tsukadoensis AD9 [35] whose functions are unknown. Searches found several homologous bacterial proteins with more than 30% identity, but the findings are annotated as putative or hypothetical proteins (Fig. 3B). MphX shows little identity (15% or less) with members of known regulator families (data not shown). Therefore, if MphX is a repressor, it can be classified as a new type of regulatory protein.

Sequence analysis of the intergenic region between mphR and mphK and determination of the transcriptional start site for mphK

As shown in Fig. 4A, in the intergenic region between mphR and mphK, three incomplete inverted repeat sequences (IR1, IR2, and IR3) [36], a long A/T-rich sequence (spanning from −134 to −29 relative to the putative transcriptional start site for mphK), and a putative σ^54-dependent −12/−24 promoter sequence (5'−TGGCATAN_{5}−TTGTA−3', N = any base) for mphK were found. The putative promoter sequence is very similar to the consensus sequence of the −12/−24 promoters (5'−TGGGACNG_{4}TTGCA−3', Y = C or T, W = A or T) described by Wigneshweraraj et al. [37]. To identify the transcriptional start site for mphK, primer extension analysis was performed using total RNA isolated from phenol-induced PHEA-2 cells. A single band corresponding to a G residue located 39 bp upstream from the translation initiation codon of mphK was detected (Fig. 4B). Considering the location of the −12/−24 promoter for mphK, this position seems suitable for the transcriptional start site for mphK.

In contrast, a putative σ^70-dependent −10/−35 promoter sequence for mphR (5'−ATGAAG−N_{27}−ATTAT−3') was also detected (Fig. 4A). This sequence is similar to the σ^70-dependent promoter (5'−GTGAGA−N_{15}−TAATAT−3') of the A. calcoaceticus NCIB8250 mphR gene [22], suggesting that mphR is transcribed from the σ^70-dependent promoter. The arrangement of mphR- KLMNOP and the above promoter/operator elements are nearly identical to those of several other phenol degradation gene clusters with a XylR/DmpR-type regulatory gene, such as dmp, mop, phe, aph, phl (strain H) and pbb [7,19,20,21,22,23].

Regulation of the transcription of mphKLMNOP, mphR, and mphX

To evaluate the transcriptional activities of P_{mphK}, P_{mphR} and P_{mphX} under various conditions, P_{mphK}, P_{mphR}, and P_{mphX}-lac^Z fusions were constructed in pGD926 and the resulting plasmids, pGDP, pGDRP and pGDPR, were independently introduced into the wild-type strain PHEA-2, the mphR-deletion mutant A2R and the mphX-deletion mutant A2X. After 6-h incubation with or without phenol, cell suspensions were prepared and β-galactosidase activity was measured as transcriptional activity using the cell suspensions. The result is summarized in Table 1. From the result, transcriptional regulation of these key mph genes can be explained as follows:

(i) Transcription of mphK (the mphKLMNOP operon): Phenol-induced PHEA-2 cells showed 10 times higher P_{mphK} activity than that of uninduced PHEA-2 cells, indicating that the transcription of mphK was phenol-inducible in PHEA-2. Irrespective of induction conditions, however, mphR deletion resulted in complete loss of P_{mphK} activity with or without phenol. This result demonstrated that mphR is essential for the transcription of mphK and encoded an activator protein. In contrast, the P_{mphK} activity of phenol-induced A2X cells was 2.4 times higher than that of the phenol-induced PHEA-2 cells, suggesting that mphX partially represses the mphR-dependent activation in PHEA-2 cells.

(ii) Transcription of mphR: Phenol-induced PHEA-2 cells showed 1.8 times higher P_{mphR} activity than that of uninduced PHEA-2 cells. This indicates that the transcription of mphR was weakly inducible in the presence of phenol. However, since a considerable level of the P_{mphR} activity was detected even in uninduced cells, a small amount of MphR is probably always produced even in the absence of effectors in PHEA-2 and may serve as a sensor for effectors coming from outside of the cells.
Low $P_{mphR}$ activity observed in both phenol-induced and uninduced A2R cells suggests that MphR is also an activator for its own gene expression. Furthermore, the absence of mphX resulted in a 40% increase in $P_{mphR}$ activity (calculated from comparison of the $P_{mphR}$ activity in phenol-induced A2X cells with that in phenol-induced PHEA-2 cells), suggesting that mphX also represses the transcription of mphR in PHEA-2 cells. This result is in good agreement with the result of the measurement of the phenol-oxygenating activities shown above.

(iii) Transcription of mphX: Phenol-induced of PHEA-2 cells showed a high level of the $P_{mphX}$ activity compared to that of uninduced PHEA-2 cells. However, in the absence of mphR or mphx (in A2R or in A2X), $P_{mphX}$ activities were at a basal level even under induction by phenol. These results indicate that the transcription of mphx was phenol-inducible and absolutely dependent on the presence of mphR and mphx itself. Very low $P_{mphX}$ activity in A2X cells even in the presence of mphR and phenol demonstrates that mphx might be in a transcriptional unit separate from mphKLMONP.

Binding of MphR to the mphR-mphK intergenic region

As shown in Fig. 3A, a region spanning IR2 and IR3 appears to contain the upstream activating sequences (UASs) of $P_{mphx}$ because it is similar to those found in the promoter regions of the mphKLAMOP, dmpKLAMOP, and xyl upper pathway gene clusters [22]. In order to investigate the direct interaction of MphR with the intergenic region between mphR and mphK, we designed a DNA fragment P380 completely covering the mphK promoter region from positions -326 to +49 relative to the transcription start site (Fig. 4A). The fragment was mixed with the purified His-MphR (Fig. 5C). Then, the mixtures of DNA/MphR complex and free DNA were resolved by native PAGE. The purified His-MphR protein with P380 caused a band shift of P380. As the amount of protein increased, shifted band was detected, indicating that the MphR protein specifically retards DNA fragments containing the mphR-mphK promoter region (Fig. 5B). This observation provides evidence for a direct interaction of MphR with the mphR-mphK promoter region, suggesting an activation mechanism similar to that proposed for a well-studied XylR/DmpR-type regulator MopR in the phenol-degrading bacterium A. calcoaceticus NCIB8250 [22].

Discussion

The expression of phenol catabolic pathway is tightly regulated by one or more regulatory proteins. One of the most extensively studied activators of phenol degradation is the plasmid-borne DmpR from Pseudomonas sp. Strain CF600 [17]. DmpR-type
Regulators consist of four functional domains: the N-terminal signal reception domain (A-domain), the central activation domain with ATPase activity (C-domain), the C-terminal DNA-binding domain with a helix-turn-helix (HTH) motif (D-domain), and a short flexible linker domain between the A-domain and the C-domain (B-domain) [27,38]. Multiple alignment of MphR with XylR/DmpR-type regulators showed that MphR has an NTP-binding motif (GxxGxGKExxAxxxHxxS, where x is any amino acid, aa 272–aa 289) [15,39] and an AAA+ protein family signature (GAYTGA, aa 319–aa 324) [40] with one amino acid difference (the complete motif is GAFTGA) in the C-domain. The former is involved in the ATP binding and hydrolysis on the C-domain.

Figure 4. Analysis of the promoter region and transcriptional start site for mphK. (A) Intergenic region between mphR and mphK. The first nucleotide in the transcript is shown in bold. Arrows and dashed arrows indicate incomplete inverted repeats (named IR1, IR2, and IR3) and the translational start codons for mphR and mphK, respectively. Double-underlines mean the putative σ^70-dependent −12/−24 promoter consensus sequence and the σ^70-dependent −10/−35 promoter consensus sequences. An arrowhead with +1 indicates the transcriptional start site for mphK. A grey-shaded box indicates the putative ribosome-binding site for the mphK transcription. (B) Mapping of the transcriptional start site for mphK by primer extension. A sequence ladder was generated with the same primer (lanes T, A, C, and G).

doi:10.1371/journal.pone.0017350.g004

DmpR-type regulators showed that MphR has an NTP-binding motif (GxxGxGKExxAxxxxHxxS, where x is any amino acid, aa 272–aa 289) [15,39] and an AAA+ protein family signature (GAYTGA, aa 319–aa 324) [40] with one amino acid difference (the complete motif is GAFTGA) in the C-domain. The former is involved in the ATP binding and hydrolysis on the C-domain,
while the latter is expected to interact with $\sigma^{54}$-RNAP. Recently, the molecular mechanism of ATP hydrolysis-mediated transcriptional activation was discussed based on the structures of bacterial enhancer-binding proteins PspF and NtrC by Rappas et al. [41]. These proteins have several common amino acid residues important in ATP binding and hydrolysis (K42, E43, N64, D107, E108, and R227 in PspF) and they are all conserved in MphR as K278, E279, N300, D343, E344, and R462, respectively. In the B-domain of MphR, a heptad-repeats signature-like sequence with regularly spaced hydrophobic amino acids is present (MxxxLxxLxxx-LxxLxxxSxxY, aa 213–aa 239), which is involved in dimer formation and prevention of ATP hydrolysis without effector binding [42]. Moreover, the HMMPFAM motif search program (http://hmmpfam.ddbj.nig.ac.jp/top-e.html) predicted the presence of a HTH DNA binding motif in the D-domain (aa 514–aa 555) of MphR. Conservation of these functionally important motifs demonstrates that MphR has many structural and mechanistic features common in NtrC family regulators including XylR/DmpR subclass members and the mechanism for the transcriptional activation by MphR is essentially identical to the above-mentioned mechanism by XylR/DmpR-type regulators.

Although some lines of experimental evidence confirming the repressor function of MphX have been obtained in this study, MphX shows little similarity with the members of known bacterial

### Table 1. β-Galactosidase activities of mphK promoter-, mphR promoter-, or mphX promoter-lacZ transcriptional fusion in A. calcoaceticus PHEA-2 and mphR- and mphX-deletion mutants (A2R and A2X).

|                | +Phenol | -Phenol |
|----------------|---------|---------|
| PHEA-2(P<sub>mphK-lacZ</sub>) | 336±33   | 34±2    |
| A2R(P<sub>mphK-lacZ</sub>)   | *       | *       |
| A2X(P<sub>mphK-lacZ</sub>)   | 807±58   | 30±2    |
| PHEA-2(P<sub>mphR-lacZ</sub>) | 160±20   | 92±10   |
| A2R(P<sub>mphR-lacZ</sub>)   | 20±5     | 21±3    |
| A2X(P<sub>mphR-lacZ</sub>)   | 225±33   | 176±15  |
| PHEA-2(P<sub>mphX-lacZ</sub>) | 332±50   | *       |
| A2R(P<sub>mphX-lacZ</sub>)   | *       | *       |
| A2X(P<sub>mphX-lacZ</sub>)   | *       | *       |

*Cells were grown in the presence (+) or absence (−) of 2.0 mM phenol and assayed for β-galactosidase activities as described in Materials and Methods. Values shown are means ± standard deviation of triplicate experiments.

*No detectable activity.

![Figure 5. Electrophoretic mobility shift assay.](A) Comparison of the upstream region of the mphK promoter with the corresponding regions of the mop gene cluster of *A. calcoaceticus* NCIB8250, the xyl upper gene cluster of *Pseudomonas putida* mt-2 (TOL), and the dmp gene cluster of *Pseudomonas* sp. CF600. Grey-shaded boxes show the putative upstream activating sequences (UASs) in the mph, mop, xyl, and dmp gene clusters to which the corresponding XylR/DmpR-type regulators bind. (B) gel retardation analysis of His-MphR binding to the mphR-mphK intergenic region. Gel-mobility shift assay was performed as described in the text using a 380-bp fragment (P380) covering the mphR-mphK intergenic region. Lane 1 is a control lane containing 2.5 nM of labeled P380 fragment only. Lane 2–7 contains 2.5 nM of the labeled P380 fragment incubated with 5, 10, 20, 30, 40 and 50 nM His-MphR protein. Lane 8 is containing 2.5 nM of labeled DNA fragment amplified using two primers, IRDye-labeled M13-F and M13-R, and plasmid pGEM T-vector as template, named P150. Lane 9–10 contains 2.5 nM of the labeled P150 fragment incubated with 30 and 50 nM His-MphR protein, respectively. The positions of the free DNA and the MphR-DNA complex are indicated. (C) SDS-PAGE of purified His-MphR protein from *E. coli* BL21 (pEMR). Lane 1: Molecular markers with their masses indicated; Lane 2: Uninduced of BL21 (pEMR); Lane 3: BL21 (pEMR) induced by 0.1 mM IPTG; Lane 4: BL21 (pEMR) induced by 0.5 mM IPTG; Lane 5: MphR purified by NTA resin and eluted by NTA-60; Lane 6: MphR purified by NTA resin and eluted by NTA-80.

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doi:10.1371/journal.pone.0017350.g005

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![Figure 5. Electrophoretic mobility shift assay.](B)

![Figure 5. Electrophoretic mobility shift assay.](C)
regulator families. BLAST (http://blast.ddbj.nig.ac.jp/top-e.html) and CDART (http://www.ncbi.nlm.nih.gov/Structure/lexington/) searches suggest that MphX belongs to the COG4313 family in the COG (Clusters of Orthologous Group) database [43]. Although most members of the COG4313 family are annotated as hypothetical or putative proteins, several proteins are designated as “involved in meta-pathway of phenol degradation”, including those encoded by orfY (identity with the encoded protein: 27.8%) in the phenol degradation gene cluster of C. testosteroni TA441 [34], orf2 (27.0%) in the aniline degradation gene cluster of P. putida UCC22 [44], and orfU (26.3%) in the aniline degradation gene cluster of D. tsukahatensis AD9 [35]. In the phenol degradation gene cluster of TA441 (aphKLMNOPQBenXtyphCCEFGRHJ), orfX is located downstream of a catechol 2,3-dioxygenase gene (aphD) [34]. Arai et al. [34] pointed out that the orfX gene product (OrfX) has a typical amino-terminal signal sequence for membrane translocation, suggesting that OrfX is a protein in the periplasm. Sequence analysis by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) predicted that MphX also has a signal peptide sequence at its amino-terminus (aa 1–aa 23, signal peptide probability = 1.000) and its most likely cleavage site (A21F22A25–T24E25, maximum cleavage site probability = 0.999). Thus, MphX has at least one feature specific to periplasmic proteins, but at present the actual location of the MphX protein in PHEA-2 is not known. The same authors found that an orfXY mutant of TA441 grew poorly on phenol and accumulated 2-hydroxyxymuconic semialdehyde [34]. To our knowledge, this is the only description of an MphX homolog-coded gene function. The mutant phenotype might be caused by a mutation in orfX, but there are also other possibilities, i.e., a mutation in orfY or a polar effect for the expression of the downstream meta-cleavage pathway genes. In the aniline degradation gene clusters of D. tsukahatensis AD9 and P. putida UCC22, MphX homolog-coded genes, orfU and orf1, are also located between two sets of genes encoding a ferredoxin-like protein and a catechol 2,3-dioxygenase (tdnD1C-tdnD2C2 and tdnD1C-tdnD2C2) [35,44]. However, the functions of these genes remain unknown. Several MphX homolog-coded genes have been found near alcohol dehydrogenase genes, such as orf1 near the 4-nitrobenzoate degradation gene cluster of Pseudomonas sp. TW3 [45], qhdB in the short-chain alcohol degradation gene cluster of P. putida HK5 [46], and chxU in the cyclohexanol degradation gene cluster of Acinetobacter sp. SE19 [47]. These gene products showed 21–22% aa sequence identity with MphX. However, the functions of these genes have not been elucidated yet. Accordingly, MphX is the only protein in this COG4313 family whose function has been demonstrated as a repressor. We looked for DNA binding motifs in MphX using several motif search programs (e.g., http://motif.genome.jp/), but we were unable to detect any HTH motif or other motifs specific to regulatory proteins in MphX.

Comamonas testosteroni strains TA441 and R5 have their respective mPH-encoding operon (aphKLMNOPQ and phcKLMNOPQ) and the counterpart XyIR/DmpR-type regulatory gene (aphR and phcR) is in almost the same gene arrangement as that of PHEA-2 [29,34]. However, strain TA441 has an additional regulatory gene, aphS, just downstream of aphR, whereas strain R5 has two additional regulatory genes, phcS and phcT, downstream of phcR. MphX shows little sequence similarity with these regulatory proteins. In strain TA441, aphS completely represses the transcriptional activation of aphKLMNOPQ caused by aphR in the presence of phenol. Consequently, strain TA441 is unable to grow on phenol, but its aphS-deficient mutant is able to grow on phenol due to the de-repression [7]. Interestingly, it was found that the transcription of aphKLMNOPQ is also caused by a gratuitous inducer, acetate, and that aphS represses this gratuitous activation, suggesting that aphS prevents wasteful expression of aphKLMNOPQ induced by such a ubiquitous carbon source [7]. In strain R5, phcS also represses the transcriptional activation of phcKLMNOPQ caused by phcR in the presence of phenol and similar gratuitous expression by acetate [29]. However, the phc gene cluster contains another regulatory gene, phcT, and the presence of phcT enabled strain R5 to overcome the repression caused by phcS and to grow on phenol [28]. The alignment analysis of these regulatory gene products indicated that AphS and PhcS belong to the GntR regulator family [7,29] while PhcT belongs to the AraC/XylS regulator family [28]. One of the AphS binding sites (consensus motif, TATCTGAT) determined by gel-mobility shift assay is located around the putative IHF (Integration Host Factor)-binding site, but not within the putative UASs [7]. Similar binding motifs were also found in the putative IHF-binding site of the phcR-phcK intergenic region [28]. Hence, it is suggested that these proteins could bind to the putative IHF binding sites and hamper the binding of IHF [15,28,48]. However, the IHF-binding consensus motif is absent in the A+T-rich sequence of the mph operon of strain PHEA-2. In particular, we were unable to detect any HTH motifs in the MphX protein using several motif search programs (e.g., http://motif.genome.jp/). Moreover, all attempts to perform electrophoretic mobility shift analysis using MphX have failed. So far we have not succeeded in the purification of MphX due to its easily-aggregating property. We presume that the mechanism by which MphX partially represses mphKLMNOPQ transcription differs from those used by AphS and PhcS; however, regulatory mechanism of the mphX gene as a repressor requires further investigation.

Most regulatory proteins of aromatic catabolic pathways act as transcriptional activators and regulation by a repressor is generally rare in aromatic compound catabolism [26,27]. The use of repressors in the regulation of phenol degradation is not yet fully understood. In the case of MphX, the most likely reason is to prevent the wasteful expression of the mPH genes, as previously described for aphS [7]. However, we have not yet found such wasteful expression induced by ubiquitous carbon sources in the absence of phenol in PHEA-2. Another reason may be to prevent excess expression of the mPH genes in the presence of phenol. This would result in the accumulation of toxic intermediates, such as catechol and muconate in cells if the expression of downstream pathway genes does not respond to that of the mPH genes. Such accumulation could potentially lead to loss of the ability to degrade aromatic compounds. This has been shown for plasmid-dependent carbazole degradation in Pseudomonas fluorescens P10-1 where catechol accumulation caused the loss of carbazole-degrading ability [49]. In addition, overproduction of one of the mPH polypeptides (MopN) was reported to cause significant growth inhibition in Acinetobacter [50]. However, in the case of PHEA-2, as shown in Fig. 2, the mphX-deletion mutant showed faster phenol degradation and better growth on phenol than the wild-type strain, indicating no toxic or inhibitory effects derived from the phenol concentration used. A similar situation was observed even at high concentrations of phenol (up to the degradation limit, 8 mM) (data not shown). Thus, this reason does not seem to be the case. It seems likely that PHEA-2 acquired the mph gene cluster through lateral gene transfer from a toxic intermediate (or over-expression)-sensitive strain. Further elucidation of the presence of this repression system and its mechanisms may provide substantial information regarding bacterial survival strategies in natural and polluted environments with phenolic compounds.
Materials and Methods

Bacterial strains, plasmids, primers, and growth conditions used

The bacterial strains and plasmids used in this study are listed in Table 2 and the sequences of all the primers used are listed in Table 3. Acinetobacter strains were grown at 30°C in mineral salts (MS) medium (NaNO₃ 0.5 g, K₂HPO₄ 0.65 g, KH₂PO₄ 0.17 g, MgSO₄ 0.10 g per liter) supplemented with phenol or sodium lactate, or in Luria-Bertani (LB) medium. *Escherichia coli* strains were grown at 37°C in LB medium. When necessary, ampicillin (20 µg/ml), kanamycin (50 µg/ml), and tetracycline (50 µg/ml) were added to the medium.

Construction of mphR-, mphN- and mphX-deletion mutants

In order to disrupt key genes in phenol degradation, the upstream and downstream regions of mphR, mphN, and mphX were generated by PCR using the total DNA of PHEA-2 as the template and several primer sets shown in Table 3. The amplified PCR products were purified and ligated into the pMD18-T vector (Takara). The resulting plasmids were double-digested with EcoRI and Sall (for plasmids carrying the upstream region) or Sall and BamHI (for those carrying the downstream region) and purified. The upstream and downstream fragments for each gene were cloned together into the EcoRI/BamHI sites of pK18mobsacB [51]. The resulting plasmids were introduced into *E. coli* JM109 and then transferred into PHEA-2 by triparental mating [52]. Integration of the introduced plasmid into the chromosome by the first crossover was selected on LB plates containing kanamycin (25 µg/ml) and ampicillin (20 µg/ml). The second crossovers were selected by culture on LB plates containing 10% (w/v) sucrose and ampicillin (20 µg/ml). The selected cells were analyzed by PCR to confirm that gene replacement had occurred (data not shown). The resulting mphR, mphN and mphX deletion mutants were designated as A2R, A2N and A2X, respectively.

Phenol degradation test

Acinetobacter strains were grown overnight in LB medium and the cultures were harvested by centrifugation (8,000×g, 4°C, 5 min). The cells were washed twice with MS medium and suspended at an OD₆₀₀ of 0.15 with MS medium containing 2 mM phenol. Cell suspensions were further incubated with shaking at 200 rpm on a rotary shaker. Aliquots of the cultures were sampled at specific intervals and centrifuged (13,000×g, 4°C, 1 min) to remove the cells. Following cell removal, phenol concentrations in the supernatants were determined using the 4-aminoantipyrine colorimetric method. This analysis was performed according to the procedures described in Folsom et al. [1].

Assay for phenol-oxygenating activity

Acinetobacter strains were grown in LB medium to the late-log phase and the cultures were harvested by centrifugation (8,000×g, 4°C, 5 min). The cells were washed twice with MS medium, suspended at an OD₆₀₀ of 0.7 with MS medium containing 2 mM phenol or 5 mM sodium lactate and further incubated at 30°C for 6 h for induction. Then, the cells were again collected by centrifugation, washed with MS medium, and resuspended at an OD₆₀₀ of 1.5 in 40 mM potassium phosphate buffer (100 mM, pH 7.5). After measurement of endogenous oxygen uptake for 5 min, reactions were started by adding phenol to the cultures to a final concentration of 0.1 mM. Oxygen concentrations in the cultures were monitored at 25°C for 3 min using a YSI 5000...
Acinetobacter strains. The resulting plasmids with the lacZ vector, pGD926, with a promoterless into the BamHI/HindIII site of a RK2-based broad host range with BamHI and HindIII. The digested fragments were ligated Madison, USA). The resulting plasmids were double-digested the template and primer sets shown in Table 3. PCR products genes were generated by PCR using the total DNA of PHEA-2 as respectively.

Overnight cultures were washed twice with MS medium induction. incubated for 6 h with shaking at 200 rpm on a rotary shaker for from three independently grown cultures was measured as moles of O2 uptake per min per gram of dry cell. The final data were obtained from three independent experiments and shown as mean ± standard error.

### Construction of plasmids containing mphR, mphN and mphX promoter-lacZ transcriptional fusion

The construction of mphR or mphX promoter-lacZ transcriptional fusion was similar to that of P_{mphk-lacZ} fusion [33,53]. The flanking region and the 5'-end region of the mphR and mphX genes were generated by PCR using the total DNA of PHEA-2 as the template and primer sets shown in Table 3. PCR products were purified and ligated into the pGEM-T vector (Promega, Madison, USA). The resulting plasmids were double-digested with BamHI and HindIII. The digested fragments were ligated into the BamHI/HindIII site of a RK2-based broad host range vector, pGD926, with a promoterless lacZ gene [52] in order to develop promoter-lacZ fusions and to introduce them into Acinetobacter strains. The resulting plasmids with the P_{mphR} or P_{mphX-lacZ} fusion were designated as pGDRP and pGD PX, respectively.

### β-Galactosidase assay

For the measurement of β-galactosidase activity in Acinetobacter strains, overnight cultures were washed twice with MS medium and suspended at an OD600 of 0.2 with MS medium containing 5 mM sodium lactate with and without 2 mM phenol and further incubated for 6 h with shaking at 200 rpm on a rotary shaker for induction. β-Galactosidase activity of cell suspensions prepared from three independently grown cultures was measured as described by Miller [54] and the values obtained were averaged and expressed in Miller units (U).

### Primer extension analysis

In order to map the transcriptional start site for mphK, the total RNA of PHEA-2 was isolated from mid-log phase cells grown in LB medium containing 1 mM phenol using a RNAspin Mini kit (GE Healthcare Bio-Sciences, Tokyo, Japan) according to the supplier’s instructions. For primer extension, an oligonucleotide labeled with FAM at its 5’ end (named Pk-FAM, 5’-GGATCCGCCACCATATGATTG-3’), and SalI (5’GGATCC-3’) were shown in bold and italic.

**Table 3. Primers used in this study.**

| Name            | Sequence (5’-3’) * | Purpose                                      |
|-----------------|-------------------|----------------------------------------------|
| Pm-F            | AGGAATTCCTTTAACCGGAGAATGCC | Amplification of mphR promoter region         |
| Pm-R            | GGATCCGAGACGTCCTGCTGATTTAGTCC | Amplification of mphX promoter region         |
| Pm-F            | GTAGAAGCTTGGAGAATGTCGCGCACG | Construction of mphN-deletion mutant         |
| Pm-R            | GGATCCGAGACGTCCTGCTGATTTAGTCC | Construction of mphX-deletion mutant         |
| X1-F            | GCAAGACTCTGACTTATCTG | Amplification of the 380-bp mphR-mphK intergenic region |
| X1-R            | ATTTGGAGAACGAGACTTCTT | Amplification of the 380-bp mphR-mphK intergenic region |
| X2-F            | ATGTCGACCTTATGACGCGT | Amplification of the 380-bp mphR-mphK intergenic region |
| X2-R            | GGATCCGAGACGTCCTGCTGATTTAGTCC | Amplification of the 380-bp mphR-mphK intergenic region |
| MphR188-F       | TCGAGACGTCCTGCTGATTTAGTCC | Amplification of the 380-bp mphR-mphK intergenic region |
| MphR207-R       | GTATTCGACCTTATGACGCGT | Amplification of the 380-bp mphR-mphK intergenic region |
| M13-fwd         | CATCAACGCTGTTGAAACGAC | Amplification of the 380-bp mphR-mphK intergenic region |
| M13-rev         | GGATACACATTTACAAGG | Amplification of the 380-bp mphR-mphK intergenic region |

*Restriction sites for BamHI (5’-GGATCC-3’), HindIII (5’-AAGCTT-3’), EcoRI (5’-GAATTC-3’), and SalI (5’-GGATCC-3’) are shown in bold and italic.

[doi:10.1371/journal.pone.0017350.t003]

β-Galactosidase assay - Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, USA) and oxygen uptake rates were corrected for endogenous respiration. Phenol-oxygenating activity was expressed as μmoles of O2 uptake per min per gram of dry cell.

### Purification of histidine-tagged MphR

The full-length mphR gene was ligated into the pET-28 vector [53] and transformed into E. coli BL21 (DE3) to express the Histagged MphR. The transformants were cultured in LB medium containing kanamycin and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 h at 10°C. The cells were

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| Pm-R            | GGATCCGAGACGTCCTGCTGATTTAGTCC | Amplification of mphX promoter region         |
| Pm-F            | GTAGAAGCTTGGAGAATGTCGCGCACG | Construction of mphN-deletion mutant         |
| Pm-R            | GGATCCGAGACGTCCTGCTGATTTAGTCC | Construction of mphX-deletion mutant         |
| X1-F            | GCAAGACTCTGACTTATCTG | Amplification of the 380-bp mphR-mphK intergenic region |
| X1-R            | ATTTGGAGAACGAGACTTCTT | Amplification of the 380-bp mphR-mphK intergenic region |
| X2-F            | ATGTCGACCTTATGACGCGT | Amplification of the 380-bp mphR-mphK intergenic region |
| X2-R            | GGATCCGAGACGTCCTGCTGATTTAGTCC | Amplification of the 380-bp mphR-mphK intergenic region |
| MphR188-F       | TCGAGACGTCCTGCTGATTTAGTCC | Amplification of the 380-bp mphR-mphK intergenic region |
| MphR207-R       | GTATTCGACCTTATGACGCGT | Amplification of the 380-bp mphR-mphK intergenic region |
| M13-fwd         | CATCAACGCTGTTGAAACGAC | Amplification of the 380-bp mphR-mphK intergenic region |
| M13-rev         | GGATACACATTTACAAGG | Amplification of the 380-bp mphR-mphK intergenic region |

*Restriction sites for BamHI (5’-GGATCC-3’), HindIII (5’-AAGCTT-3’), EcoRI (5’-GAATTC-3’), and SalI (5’-GGATCC-3’) are shown in bold and italic.

[doi:10.1371/journal.pone.0017350.t003]
harvested by centrifugation and disrupted by sonication (160 W, 2 s bursts with a 3 s interval between every two bursts) for 10 min on ice. The lysate was centrifuged (20,000 x g, 4°C, 20 min) and the supernatant was immediately used for protein purification by the nickel-nitrilotriacetic acid (Ni-NTA) columns (Qiagen) according to the manufacturer’s recommendations. Protein concentrations were measured by the Bradford method [53].

Gel mobility-shift assay

For the gel mobility-shift assay, a fragment (390 bp) containing the intact mphR- mphK intergenic region was prepared (Fig. 4A). First, the intergenic region was amplified by PCR using two primers, MphR188-F and MphK207-R (Table 3), and PHEA-2 total DNA as the template. Then, the amplified fragment was purified and ligated into the pGEM-T vector (Promega). The resulting construct was designated as pGE380 (Table 2). The sequence of the insert fragment was confirmed by sequencing. Secondly, the intergenic region was amplified again using a different primer set (M13-fwd and M13-rev) labeled with IRD800 infrared dye at the 5’ end (Table 3) (Li-cor) and pGE380 as the template. At the same time, the control DNA fragment was amplified using the same primer set and the pGEM-T vector as

the template. The amplified fragment was electrophoretically purified from a 2% (w/v) low temperature-melting agarose gel (Cambrex, East Rutherford, USA) according to the protocol and named P380. Binding reaction mixtures contained 20 mM Tris-HCl [pH 7.5], 50 mM KCl, 6 mM DTT, 0.5% (v/v) Tween-20, 0.05 μg/ml poly (dl-IC) (Roche, Mannheim, Germany), 2.5 nM DNA fragment and the purified histidine-tagged (His-) MphR with the increasing concentrations in a final volume of 10 μl. After incubation for 20 min at 30°C in darkness, 1 μl of 10× Orange loading dye (0.02 g orange G, 6 ml 50% [v/v] glycerol, 1.2 ml 0.5 M EDTA [pH 8.0], 2.87 ml sterile water) was added to the mixtures, mixed briefly, and then loaded onto a 8% polyacrylamide gel. Electrophoresis was carried out at 10 V/cm for about 3 h in 0.5× TBE buffer (45 mM Tris borate, 1 mM EDTA) in darkness. Scanning of the gel was conducted using an Odyssey Imager (LI-COR) under the supplier’s recommended condition.

Author Contributions

Conceived and designed the experiments: ML. Performed the experiments: HY ZP YZ ZZ SI, JW YY MC. Analyzed the data: JW YY MC WL SP. Wrote the paper: HY ZP MT ML.

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