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Aromatic Effector Activation of the NtrC-Like Transcriptional Regulator PhhR Limits the Catabolic Potential of the (Methyl)Phenol Degradative Pathway It Controls

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Pseudomonas putida* P35X (NCIB 9869) metabolizes phenol and monomethylphenols via a chromosomally encoded *meta*-cleavage pathway. We have recently described a 13.4-kb fragment of the chromosome that codes for the first eight genes of the catabolic pathway and a divergently transcribed positive regulator, *phhR*. The eight structural genes lie in an operon, the *phh* operon, downstream of a −24 TGGC, −12 TTGC promoter sequence. Promoters of this class are recognized by RNA polymerase that utilizes the alternative σ^{54} factor encoded by *rpoN* (*ntrA*) and are positively regulated by activators of the NtrC family. In this study, we have identified the coding region for the 63-kDa PhhR gene product by nucleotide sequencing of a 2,040-bp region and polypeptide analysis. PhhR was found to have homology with the NtrC family of transcriptional activators, in particular with DmpR, the pVI150-encoded regulator of (methyl)phenol catabolism by *Pseudomonas* sp. strain CF600. By using a luciferase reporter system, PhhR alone was shown to be sufficient to activate transcription from the *phh* operon promoter in an $RpoN^+$ background but not an $RpoN^-$ background. Luciferase reporter systems were also used to directly compare the aromatic effector profiles of PhhR and DmpR. Evidence that the difference in the growth substrate ranges of strains P35X and CF600 is due to the effector activation specificities of the regulators of these systems rather than the substrate specificities of the catabolic enzymes is presented.

Pseudomonas putida P35X can degrade phenol and monomethylated phenols by virtue of a chromosomally encoded pathway (10, 13). The biochemical route for dissimilation of these compounds involves a multicomponent phenol hydroxylase and a subsequent *meta*-cleavage pathway. The nucleotide sequences of the first eight genes of this pathway have previously been determined and found to lie in an operon structure designated the *phh* operon (19). The deduced amino acid sequences of these gene products have a high (68 to 99%) degree of identity with isofunctional proteins of the (methyl) phenol degradative pathway encoded by the plasmid-located *dmp* operon of *Pseudomonas* sp. strain CF600 (26).

Nucleotide sequence analysis also identified a putative −24, −12 promoter sequence 31 bp from the ATG initiation codon of the first gene of the *phh* operon (19). Here we report the nucleotide sequence of the *phhR* regulator whose product controls transcription of the *phh* operon. PhhR has homology to members of the NtrC family of transcriptional activators which control a variety of physiological processes in response to environmental signals (reviewed in references 15, 16, and 20). This family of regulators control transcription from −12, −24 promoters recognized by RNA polymerase that utilizes the alternative σ^{54} factor encoded by *rpoN* and its analogs and usually bind to large inverted repeats located 120 to 180 bp upstream from the promoters they control. Within these systems, close proximity between the upstream bound regulator and the promoter-bound RNA polymerase-σ^{54} complex is mediated by DNA loop formation that is often aided by binding of the DNA bending protein IHF.

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Members of the NtrC family of regulatory proteins have distinct functional domains defined on the basis of the most extensively studied members, namely, NtrC and NifA (5). The central C domain is the most conserved within the family and probably mediates interaction with RNA polymerase and the binding and hydrolysis of ATP for formation of open transcriptional complexes. The carboxy D domains contain a helix-turn-helix DNA binding motif and also are conserved among members of the family (reviewed in reference 17). However, the regulatory amino-terminal A domains of these proteins, which are joined to the central domain by a short sequence that serves as a flexible interdomain linker (29), fall into subgroups that reflect the mechanism(s) of activation of different regulators. Most members of the NtrC family are part of so-called two-component systems which consist of a sensory histidine kinase which activates the constitutively expressed regulatory protein by transfer of a phosphate group to the regulatory A domain (reviewed in references 27 and 28). However, DmpR (24) and XylR (1, 12), two *Pseudomonas*-derived members of the NtrC family that control catabolism of aromatic compounds, appear to be directly activated by the presence of specific aromatic compounds delivered in the culture medium. The regulatory A domains of these proteins have been shown to mediate the specificity of activation in response to the aromatic compounds catabolized via the products of the genes they regulate (2, 22, 25).

PhhR was found to have extensive identity with DmpR and XylR, and this homology included the regulatory A domain. Despite the similarity of both regulatory and structural genes encoded by the *phh* and *dmp* systems, these two systems do not specify growth on the same range of substrates; while both systems mediate growth on phenol and monomethylated phenols, only the *dmp* system mediates growth on 3,4-dimethyl-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Properties	Reference or source
Strains		
E. coli		
S17-1λ*pir*	Tp^r Sm^r Res$^-$ RP4-mob$^+$ λ*pir* lysogen	4
DH5	Prototrophic, Res$^-$	9
P. putida		
P35X	(Methyl)phenol degrader harboring the 3,5-dimethylphenol catabolic plasmid pRA500	10
RA713	Plasmid-cured derivative of P35X	13
TN2100	Prototrophic, RpoN$^+$	18
TS1012	RpoN$^-$ Km^r transposon mutant of TN2100	11
KT2440	Prototrophic, Res$^-$	7
Plasmids		
pUC128	Ap^r cloning vector	14
pUT/mini-Tn*5* Km2	Ap^r Km^r, mobilizable *pir*-dependent R6K transposon delivery vector	4
pLX303-ab	Source of promoterless *Vibrio harveyi luxAB* genes carried on a 2.35-kb *Bam*HI fragment	21
pbs (SK+)-NotI	Stategene pBluescript (SK+) vector with an additional *Not*I site replacing the *Xho*I site	6
pMMB66EH	Ap^r, RSF1010-based broad-host-range *tac* promoter-*lacI*^{q} expression vector	8
pMMB66HE	As above, but reverse orientation of the polylinker	8
pVI398	Ap^r, pMMB66HE-based cloning vector deleted for the *tac* promoter and *lacI*^{q} gene	22
pVI400	Ap^r, pVI398-based plasmid carrying *dmpR* under control of its natural promoter	25
pVI430	Ap^r Km^r, *dmp* operon promoter controlling transcription of *luxAB* on pUT/mini-Tn*5* Km2	22
pLCN1	Ap^r, 13.4-kb *Eco*RI fragment from *P. putida* P35X involved in methylphenol catabolism	19
pLCN20	Ap^r, 3.5-kb *Sma*I fragment spanning *phhR* in pUC128	This study
pLCN23	Ap^r, pVI398-based plasmid with the 3.5-kb *Sma*I fragment spanning *phhR* and the *phh* operon promoter controlling transcription of *luxAB*	This study
pLCN24	Ap^r, 0.7-kb *Bgl*II-to-*Sma*I *phh* operon promoter fragment controlling *luxAB* on pVI398	This study
pLCN26	Ap^r, 3.5-kb *Sma*I fragment spanning *phhR* oriented so that it is under the control of the pMMB66EH *tac* promoter	This study
pLCN27	Ap^r Km^r, *phh* operon promoter controlling transcription of *luxAB* on pUT/mini-Tn*5* Km2	This study
pLCN33	Ap^r, 3.5-kb *Sma*I fragment spanning *phhR* in pVI389	This study

phenol (3,4-dmp). By comparing the aromatic effector specificity profiles of PhhR and DmpR and introducing DmpR into the *phh* system, we show that it is the effector specificity of the regulator which limits the catabolic potential of *P. putida* P35X.

MATERIALS AND METHODS

Strains, plasmids, and general procedures. The bacterial strains and plasmids used in this study are listed in Table 1, and the DNA in key constructs is illustrated in Fig. 1. *Escherichia coli* strains were grown at 37°C, and *Pseudomonas* strains were grown at 30°C. Luria broth was used as rich medium, while minimal M9 salts supplemented with a 2.5 mM carbon source (23) were used in growth tests. Broad-host-range plasmids were introduced into *Pseudomonas* strains by electroporation with a Bio-Rad Gene Pulser. R6K-based, *pir*-dependent plasmids were introduced by conjugation from S17-1λ*pir*.

DNA manipulations and nucleotide sequence determinations. DNA manipulations were performed by standard techniques (23), with *E. coli* DH5 as the host strain. Deletion libraries of pLCN20, which carries a 3.5-kb *Sma*I fragment in pUC128, were generated by using Pharmacia's double-stranded nested deletion kit. The nucleotide sequences of both strands were determined directly from plasmids by using commercial kits and the M13 universal and reverse primers from Promega. Gaps in deletion libraries were determined by using custom-designed oligonucleotides.

Polypeptide analysis. Plasmids were in vitro transcribed and translated by using an *E. coli* S30 extract system for circular templates (Promega) and [^{35}S]methionine (Amersham). The resulting radiolabelled polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the dried gel was subjected to autoradiography.

Construction of KT2440 Km-P$_h$::*luxAB* reporter strain. First, the 0.7-kb *Bgl*II-to-*Sma*I P$_h$ promoter fragment was cloned as an *Eco*RI-to-*Bam*HI fragment from pLCN24 between these sites on a pBluescript derivative, pbs (SK+)-NotI, in which an additional *Not*I site had been introduced by replacement of the *Xho*I site. The 2.35-kb *Bam*HI fragment from pLX303-ab which carried the promoterless *luxAB* genes was then inserted in the correct orientation downstream of the P$_h$ promoter. The resulting P$_h$::*luxAB* fusion was then excised as a 3.05-kb *Not*I fragment and inserted into the unique *Not*I site within the defective transposon mini-Tn*5* Km2 carried on the R6K *pir*-dependent suicide vector pUT and into the chromosome of *P. putida* KT2440 as described elsewhere (4).

Luciferase assays. *P. putida* strains that harbored test plasmids were grown overnight in Luria broth that contained carbenicillin (1 mg/ml) to select for retention of the resident plasmid. Cells were subsequently diluted 1:1,000 in the same media and grown to late exponential phase (A_{650} = 2.5). At that time, aliquots of cells were left unsupplemented or supplemented with 2.5 mM effector dissolved in dimethyl sulfoxide and further incubated with rigorous shaking for 4 h. Then luciferase assays were performed with an LKB 1250 luminometer as previously described (21).

CO_2 production measurements. Cells were grown overnight on minimal M9 salts plates supplemented with phenol or 2-methylphenol as the sole carbon source and resuspended in 0.9% NaCl to an A_{650} of 0.2. Approximately 10^6 cells were plated on 58-mm-diameter minimal M9 salts plates that contained 2.5 mM aromatic compounds. Plates were incubated at 30°C in a Respicon III respirometer (Norgren Innovation AB, Umeå, Sweden), and CO_2 production was measured every 30 min for up to 45 h.

Nucleotide sequence accession number. The 2,040-bp sequence spanning *phhR* has been deposited in the EMBL database under accession number X79599.

RESULTS AND DISCUSSION

PhhR mediates transcription of *phh* operon promoter. Previous determinations of the nucleotide sequences of the first eight genes of the catabolic operon allowed unambiguous location of the *phhKLMNOPQB* structural genes on the 13.4-kb physical map (Fig. 1) (19). This study also demonstrated that a regulatory gene, *phhR*, was encoded upstream of the *phh* operon between the *Eco*RI and *Sma*I sites (coordinates, 0 to 3.9 kb) (Fig. 1). In addition to the structural genes of the *phh* operon, the upstream regulatory region also shows remarkable similarity (97% identity) to that of the *dmp* operon (24) (Fig. 2) and contains the following features associated with regulation of the *dmp* operon by DmpR: (i) a σ^{54} (*rpoN*)-dependent −24, −12 promoter sequence 31 bp upstream from the ATG start of the first gene of the operon, (ii) a large inverted repeat believed to be the binding site for the regulator, and (iii) a sequence homologous to the binding site for IHF that overlaps the long inverted repeat (24). Since −24, −12 promoters are dependent on σ^{54} (RpoN), PhhR-mediated transcription from

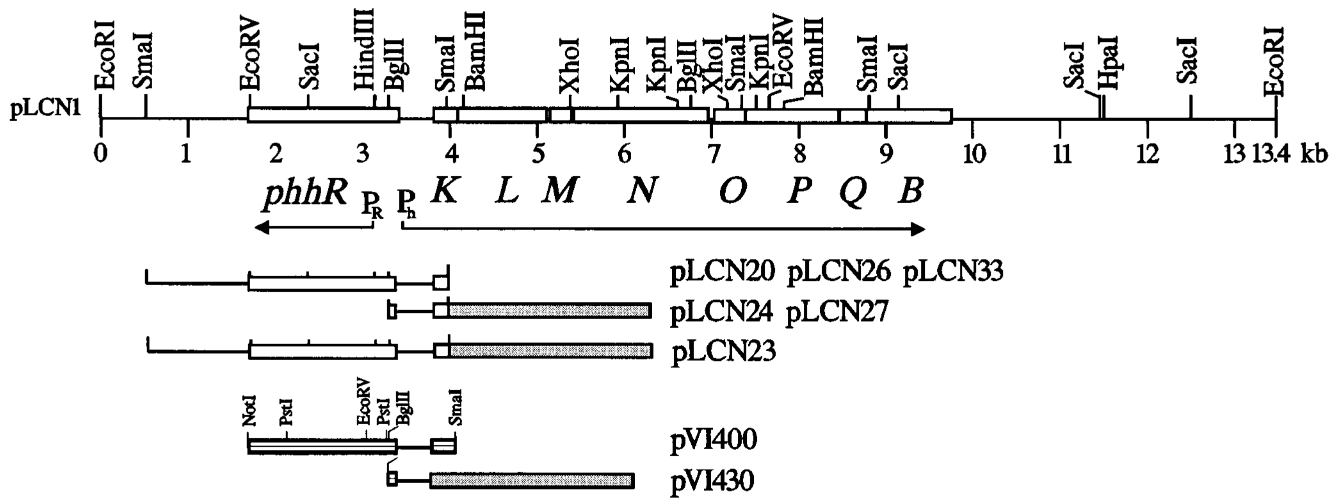

FIG. 1. Restriction map of the 13.4-kb *Eco*RI fragment of pLCN1 and key constructs used in this study. The locations of the *phhR* regulatory gene, the *phhKLMNOP* genes that encode a multicomponent phenol hydroxylase, and the *phhQB* genes involved in catechol-2,3-oxygenase activity (19) are indicated by open boxes. Striped boxes indicate the extent of DNA encoding DmpR, while stippled boxes indicate the locations and extent of promoterless *luxAB* genes.

the P_h promoter of the *phh* operon was analyzed in both $RpoN^-$ and $RpoN^+$ backgrounds. For this purpose, we generated two reporter plasmids, pLCN23 and pLCN24, based on the broad-host-range plasmid pVI398, which has no promoter directed toward the polycloning cassette (Table 1). Plasmid pLCN24 carries the promoterless *luxAB* genes downstream of the P_h promoter region, while pLCN23 carries the same DNA in addition to the *phhR* gene in its native configuration relative to P_h (Fig. 1). Both pLCN23 and pLCN24 were introduced into *P. putida* TN2100 ($RpoN^+$) and a transposon insertion derivative of TN2100, *P. putida* TS1012 ($RpoN^-$). Transcription from the P_h promoter in resulting strains in the presence or absence of phenol was monitored by measuring luciferase activity. The results shown in Table 2 demonstrate that (i) PhhR is necessary and sufficient for transcription from the P_h promoter region, (ii) transcription is dependent on the presence of phenol, and (iii) transcription is observed only in the $RpoN^+$ background.

Nucleotide sequence and homologies of *phhR*. Nucleotide sequence analysis of the *phhR*-encoding region (see Materials and Methods) identified a 563-codon open reading frame. This region was found to encode a single polypeptide of approximately 63 kDa, which correlates well with the predicted size of 63.2 kDa for PhhR (data not shown).

Comparison of the deduced amino acid sequence of PhhR with database sequences demonstrated extensive homology for the central and carboxy regions of this protein (residues 234 to 472 and 519 to 558 of PhhR) with those of members of the NtrC family of transcriptional activators. Eleven members of this family have been compared, and associated functional motifs, all of which also are conserved in PhhR, have recently been reviewed (20). However, the amino-terminal A domain of PhhR (residues 1 to 210) was found to have significant identity only with those of DmpR (87%) and XylR (62%), two *Pseudomonas*-derived regulators which control (methyl)phenol and toluene-xylene catabolism, respectively. Two approaches have demonstrated that the A domains of DmpR and XylR are directly involved in activation of these regulators by interaction with distinct aromatic compounds. First, a chimeric protein with the A domain of DmpR substituted for that of XylR was found to completely change its aromatic effector specificity profile from that of DmpR to that of XylR (25). Secondly, mutants with single amino acid changes in the A domains of both DmpR (22) and XylR (2) which respond to aromatic compounds that are not effectors (activators) of the wild-type regulators have been isolated.

Coexpression of *dmpR* can promote efficient *phh*-mediated growth on 3,4-dmp. Given the extensive homology between the structural genes, regulatory genes, and intervening regulatory regions of the *phh* and *dmp* systems, it is surprising that these

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phhR CATGTAAGCGAGGCCCCTATTTATTTTTAGATGGGGAAA-TCAGGGTCGCCGCTATAGCGCAAGGCAGGCGGCGATTCCAGATGGGGTCATAGGGAAAATCGGCAGT 106
dmpR CATGTAAGCGAGGCCCCTATTTATTTTTAGATGGGGAAAATCAGGGTCGCCGCTATAGCGCAAGGCAGGCGGCGATTCCAGATGGGGTCATGGGGAAAATCGGCAGT 107

TTTTTCACCTGCGCCAGCTCCCCATAGCCGGCCTCAGTATCTGGCAAAAGTCAACCAAATTATCAATCGACGGCAGTGACTTTAGTACTAGAGATCAGCGTTCAGCTGCGCC 218
TTTTTCACCTGCGCCAGCTCCCCATAGCCAGCCTCAGTATCTGGCAAAAGTCAACCAAATGATCAATCGACGGCAGTGATTTTAGTATTAGAGATCAGCGTTCAGCTGCGCC 219

ATGAGCATTTGCTCAAACGGCCTTGGGCAATTGATCAAATGCTTAAAAAGTCTGCGCAAGCGCGGCTTAATTTCGCTCGCTCCGATCATTCTAAAAATTAGAAACACATTGA 330
ATAAGCATTTGCTCAAGCGGCCTTGGGCAATTGATCAAATGCTTAAAAAGTCTGCGCAAGCGCGGCTTAATTTCGCTCGCTCCGATCATTCTAAAAATTAGAAACACATTGA 331
                          5'-WATCAANNNNTTR-3' core IHF binding sequence

AAAACAATACCTTGAAGTCGGTTTTCAGAGGTTTTCAGACCTTGGCACAGCTGTTGCTTTATGTCCTGCGCAATCCGCCAACCTGGAGATG phhK of phh-operon
AAAACAATACCTTGAAGTCTGTTTTCAGATGTTTTCAGACCTTGGCACAGCCGTTGCTTGATGTCCTGCGCAAGCCGCCAACCTGGAGATG dmpK of dmp-operon
                                                                    +1
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FIG. 2. Alignment of the regulatory regions between divergently transcribed regulators and catabolic operons of the *phh* and *dmp* systems. Differences are indicated by pound signs, and ATG initiation codons are shown in italics. The locations of −12, −24 promoter sequences are indicated by combinations of bold letters and asterisks, while bold letters and discontinuous arrows indicate the locations and extents of long inverted repeats. These inverted repeats have approximately 70% identity with the binding site of XylR and probably encompass the binding sites for PhhR and DmpR (6). The locations of a previously identified putative IHF binding site and transcriptional initiation site are also indicated (24).

TABLE 2. PhhR-mediated transcription of the *luxAB* genes from P_h in $RpoN^+$ and $RpoN^-$ backgrounds

Strain and plasmid	Luciferase activity[a]	
	−Phenol	+Phenol
TN2100 ($RpoN^+$)		
pLCN23 (*phhR* P_h::*luxAB*)	81 ± 1	29,100 ± 2,100
pLCN24 (P_h::*luxAB*)	83 ± 10	77 ± 17
TS1012 ($RpoN^-$)		
pLCN23 (*phhR* P_h::*luxAB*)	19 ± 3	28 ± 6
pLCN24 (P_h::*luxAB*)	5 ± 1	8 ± 2

[a] Cells were grown in the presence (+) or absence (−) of 2.5 mM phenol and assayed for luciferase activity as described in Materials and Methods. Data are expressed as luxes per milliliter of cells at an optical density at 600 nm and are the averages of triplicate determinations of two independent experiments.

two systems do not mediate growth on the same range of aromatic compounds. While both systems mediate growth on phenol and monomethylated phenols, *P. putida* P35X and its plasmid-cured derivative RA713 cannot grow on 3,4-dmp. A previous study showed that at least the first two enzymes of the *phh* pathway were not only capable of catalyzing reactions with 3,4-dmp and its hydroxylated product but were also the preferred substrates (19). This result suggested that the limitation arose within the regulatory system rather than from the specificities of catabolic enzymes of the system.

To test if the transcriptional regulator mediated this property, a plasmid that expressed *dmpR* from its natural promoter was introduced in *trans* to the *phh* system in RA713. The resulting strain was found to have gained the ability to grow on 3,4-dmp (19). The ability of RA713, with or without the *dmpR*-expressing plasmid, to catabolize phenol, 2-methylphenol, and 3,4-dmp was measured by quantifying CO_2 production from bacteria grown on plates that contained these compounds as sole carbon and energy sources. The results summarized in Fig. 3 demonstrate that RA713(pVI400 [DmpR]) grew efficiently on 3,4-dmp, with a doubling time that was only 27 to 35% longer than those found for phenol and 2-methylphenol, while RA713 without the *dmpR* plasmid survived and continued to respire but was unable to grow on this substrate. Hence, coexpression of DmpR alleviates the restriction within the *phh* system and expands the growth substrate range of RA713 to include 3,4-dmp.

Aromatic effector specificity profile of PhhR compared with that of DmpR. The growth test results suggested that the effector specificities of PhhR and DmpR differ. To directly compare the effector specificities of these two regulators, two *luxAB* reporter systems were used. First, we constructed a reporter strain, *P. putida* KT2440 Km-P_h::*luxAB*, which carries the *phh* operon promoter-reporter gene fusion on the chromosome (see Materials and Methods). For comparison, we also used the previously constructed reporter strain *P. putida* KT2440 Km-P_o::*luxAB*, which carries the *luxAB* genes placed at the +2 position relative to the transcriptional start from the *dmp* operon promoter, P_o (Fig. 1) (22). Plasmids pLCN33 (PhhR) and pVI400 (DmpR), which express the two regulatory genes from their native promoters, were introduced into the two reporter strains. Transcription from promoters was monitored by measuring luciferase activity after exposure of strains to phenol and methylphenols. The data shown in Fig. 4 demonstrate that both regulators respond to phenol, 2-methylphenol, 3-methylphenol, and 3,4-dmp. However, the magnitude of the response varies with the position of the substituent(s) on the aromatic ring, as has been previously found for DmpR (22, 25).

In both test systems, PhhR is less efficient than DmpR in responding to all of the aromatic compounds tested, including phenol and 2-methylphenol. However, the lower transcriptional responses in these cases do not seem to limit growth rates on these compounds since coexpression of DmpR does not enhance growth (Fig. 3). Most importantly, PhhR clearly responds very poorly to the presence of 3,4-dmp. Therefore, we conclude that it is the inefficient effector activation of PhhR by 3,4-dmp that prevents growth on this aromatic substrate.

Both genetic systems used to monitor transcription have operon promoter-reporter gene fusions shielded from external transcriptional activity by the presence of strong termination signals located at the boundaries of the delivery transposon (3). Nevertheless, the absolute level of the regulator-mediated transcriptional response is dependent on the site of insertion of the transposon into the chromosome (data not shown). One possible explanation for insertion site influence is the transcriptional and supercoiled status of chromosomal DNA. As

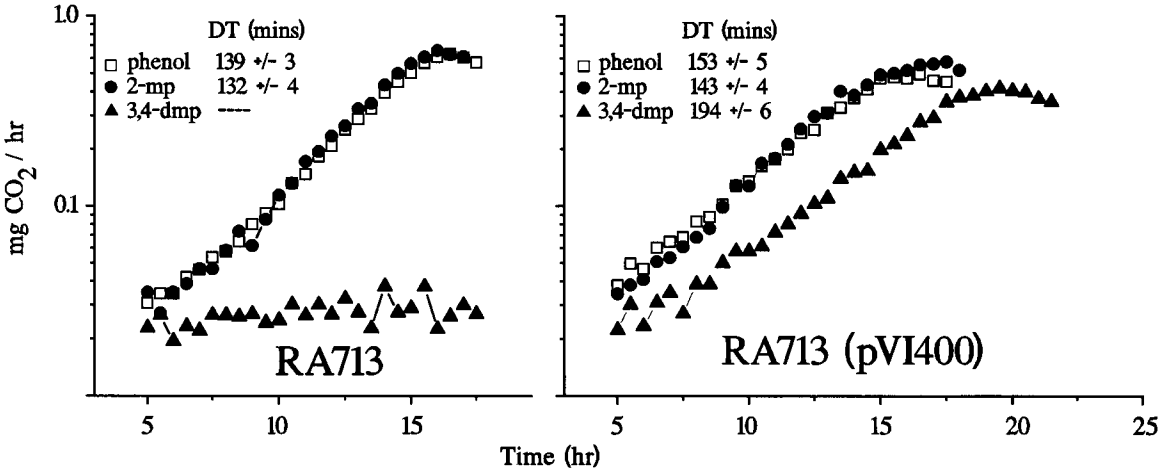

FIG. 3. Rate of CO_2 production by RA713 and RA713(pVI400 [DmpR]) upon growth with phenol, 2-methylphenol (2-mp), or 3,4-dmp as the sole carbon source. Doubling times (DT) were calculated from four independent experiments, and results from one representative experiment are shown. The presence of *dmpR* has a slightly detrimental effect on the rate at which RA713 catabolizes phenol and 2-mp that may be due to the added metabolic load of carrying the pVI400 plasmid.

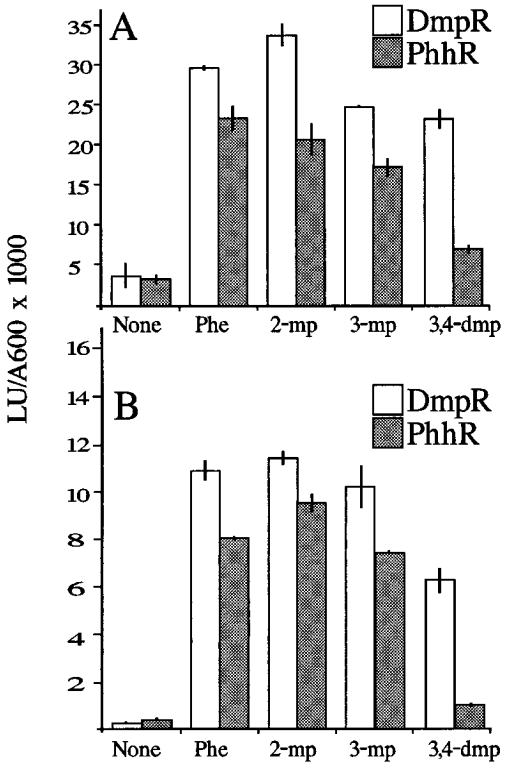

FIG. 4. Transcriptional responses (in luxes [LU] per unit of optical density at 600 nm [A_{600}] of KT2440 Km-P_h::*luxAB* (A) and KT2440 Km-P_o::*luxAB* (B) that harbor plasmids that express *phhR* (pLCN33) or *dmpR* (pVI400) from their native promoters. Cells were grown and treated as described in Materials and Methods. The responses to the presence of 2.5 mM phenol (Phe), monomethylated phenols (2-mp and 3-mp), and 3,4-dmp are shown. Data are averages of triplicate determinations in at least two independent experiments; error bars are shown. The results for pVI400 (DmpR) in KT2440 Km-P_o::*luxAB* have previously been published (22) but are shown here for comparison. Occasionally, transformants of the KT2440 Km-P_h::*luxAB* strain with pLCN33 (PhhR) or its derivatives that gave higher absolute values but the same pattern of response as shown were found (<10%). Isolation of plasmid DNA from these strains and subsequent reintroduction into the test strain was found to give approximately the same distribution of transformants as found with the original plasmid preparation. At present, we do not know the reason behind this apparent genetic instability; however, it may be relevant that the chromosomal insertion and resident plasmid share a region of 767 bp (Fig. 1). The higher transcriptional response seen with the P_h::*luxAB* strain suggests that recombination between one of the 16 to 20 copies of the plasmid and the chromosomally located insert may be involved.

can be seen in Fig. 4, the P_h::*luxAB* system produces higher basal levels and absolute values than the P_o::*luxAB* system does. In view of the high homology of the P_h and P_o regulatory regions (Fig. 2), the differences between the two systems probably reflect positional differences within the chromosome rather than intrinsic differences between the two promoters.

Concluding remarks. In this report, we have demonstrated that the effector specificity of PhhR limits the catabolic potential of the pathway it controls and that introduction of the DmpR regulator, which has broader effector specificity, expands the metabolic capacity of the strain. Little is known of the molecular mechanism by which regulators such as PhhR, DmpR, and XylR become activated by the presence of aromatic compounds. The high degree of identity between PhhR and DmpR, in conjunction with their different effector profiles in terms of response to 3,4-dmp, provides an additional tool with which to dissect the activation process. We are currently isolating effector specificity mutants of PhhR in order to pinpoint the specific amino acid residues responsible for specificity differences between these two regulators.

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