JOURNAL OF BACTERIOLOGY, May 1999, p. 2789–2796 Vol. 181, No. 9
0021-9193/99/$04.00+0

PhhB, a *Pseudomonas aeruginosa* Homolog of Mammalian Pterin 4a-Carbinolamine Dehydratase/DCoH, Does Not Regulate Expression of Phenylalanine Hydroxylase at the Transcriptional Level†

JIAN SONG,‡ TIANHUI XIA, AND ROY A. JENSEN*

Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611-0700

Received 30 November 1998/Accepted 26 February 1999

Pterin 4a-carbinolamine dehydratase is bifunctional in mammals. In addition to playing a catalytic role in pterin recycling in the cytoplasm, it plays a regulatory role in the nucleus, where it acts as a dimerization-cofactor component (called DCoH) for the transcriptional activator HNF-1α. A thus far unique operon in *Pseudomonas aeruginosa* contains a gene encoding a homolog (PhhB) of the regulatory dehydratase, together with genes encoding phenylalanine hydroxylase (PhhA) and aromatic aminotransferase (PhhC). Using complementation of tyrosine auxotrophy in *Escherichia coli* as a functional test, we have found that the in vivo function of PhhA requires PhhB. Strikingly, mammalian DCoH was an effective substitute for PhhB, and either one was effective in *trans*. Surprisingly, the required presence of PhhB for complementation did not reflect a critical positive regulatory effect of *phhB* on *phhA* expression. Rather, in the absence of PhhB, PhhA was found to be extremely toxic in *E. coli*, probably due to the nonenzymatic formation of 7-biopterin or a similar derivative. However, bacterial PhhB does appear to exert modest regulatory effects in addition to having a catalytic function. PhhB enhances the level of PhhA two- to threefold, as was demonstrated by gene inactivation of *phhB* in *P. aeruginosa* and by comparison of the levels of expression of PhhA in the presence and absence of PhhB in *Escherichia coli*. Experiments using constructs having transcriptional and translational fusions with a *lacZ* reporter indicated that PhhB activates PhhA at the posttranscriptional level. Regulation of PhhA and PhhB is semicoordinate; both PhhA and PhhB are induced coordinately in the presence of either L-tyrosine or L-phenylalanine, but PhhB exhibits a significant basal level of activity that is lacking for PhhA. Immunoprecipitation and affinity chromatography showed that PhhA and PhhB form a protein-protein complex.

Pterin-4a-carbinolamine dehydratase catalyzes the dehydration step in the cyclic regeneration of tetrahydrobiopterin (BH_4), an essential cofactor required for the phenylalanine hydroxylase reaction (Fig. 1). During catalysis, BH_4 is stoichiometrically oxidized to a 4a-hydroxytetrahydrobiopterin, which is then converted by carbinolamine dehydratase to quinonoid dihydrobiopterin. The latter compound is cycled back to BH_4 by an NADH-dependent dihydropteridine reductase (12), as shown in Fig. 1B. The importance of carbinolamine dehydratase in the regeneration of BH_4 has become apparent because mutations in the human gene which encode it result in hyperphenylalaninemia (29) and in the depigmentation disorder vitiligo (25). Affected human subjects were found to excrete large amounts of a chemically rearranged form of 6-biopterin, 7-biopterin (25). The latter is a potent inhibitor of phenylalanine hydroxylase, thereby interfering with phenylalanine catabolism and with biosynthesis of melanoid pigments. A nonenzymatic rearrangement of 6-biopterin to 7-biopterin occurs in the absence of carbinolamine dehydratase (4, 29). Thus, it appears that carbinolamine dehydratase stimulates the phenylalanine hydroxylation reaction, not only by regenerating the essential pterin cofactor but also by preventing the formation of the inhibitory 7-biopterin.

Recently, it became apparent as the result of entirely independent sequencing results that cytoplasmic carbinolamine dehydratase is synonymous with a nuclear regulatory protein, DCoH (3). DCoH stabilizes the dimeric state of HNF-1α, a homeodomain-containing protein which transcriptionally activates tissue-specific expression of a large number of genes in liver, intestine, and kidney (16). In addition, DCoH was found to be a maternal factor in rat eggs, where HNF1 transcriptional factors are absent (20). This evidence for an HNF1-independent function of DCoH was further indicated by the presence of DCoH in the eyes and the brains of rat embryos, which maintain cell types lacking HNF1 proteins (20).

In an earlier report, we identified homologs of mammalian phenylalanine hydroxylase and 4a-carbinolamine dehydratase/DCoH as two gene members of a three-component operon in *Pseudomonas aeruginosa* (34). Phenylalanine hydroxylase (PhhA) is encoded by the first structural gene (*phhA*), and carbinolamine dehydratase is encoded by the second structural gene (*phhB*) of the *phh* operon (Fig. 1A). Subclone analysis with *Escherichia coli* suggested that *phhB* was required for in vivo function of *phhA*. Thus, in the absence of the *phhB* gene, *phhA* by itself was unable to complement *E. coli* tyrosine auxotrophy. Since the mammalian counterpart of PhhB is a bifunctional protein endowed with both enzymatic activity and regulatory activity, the foregoing observation suggested that the bacterial PhhB might also have a regulatory function. This possibility has been further enhanced by recent reports of structural features shared by PhhB and DCoH. The three-

* Corresponding author. Mailing address: Department of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611-0700. Phone: (352) 392-9677. Fax: (352) 392-5922. E-mail: rjensen@micro.ifas.ufl.edu.

† Florida Agricultural Experiment Station Journal series no. R-06836.

‡ Present address: Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109-0620.

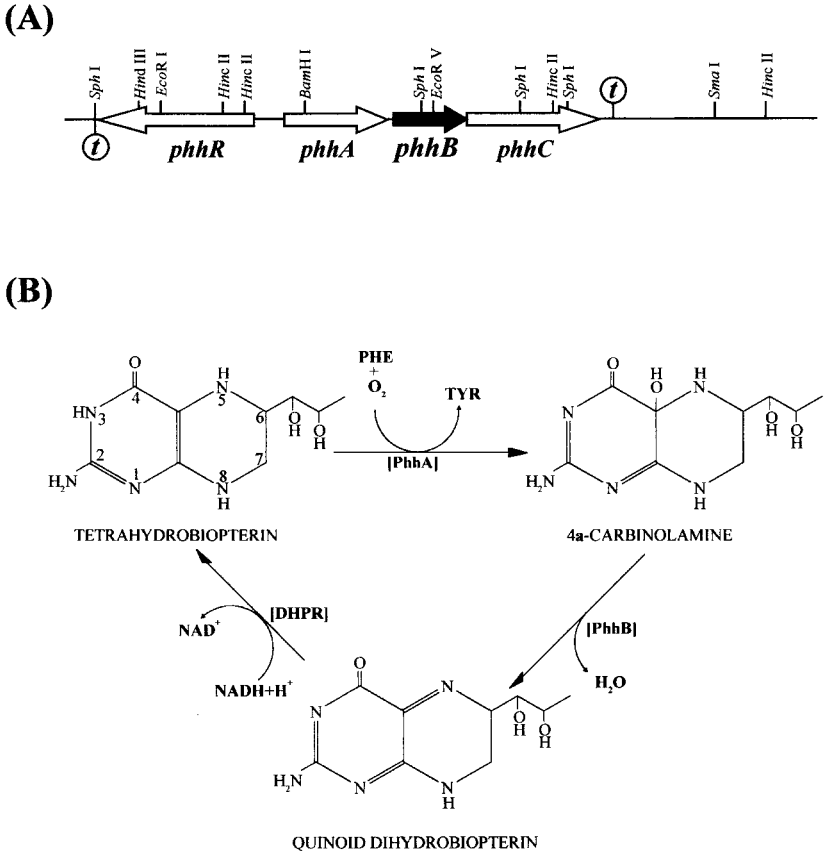

FIG. 1. (A) Physical map of the *phh* operon in *P. aeruginosa*. The endonuclease restriction sites are shown at the top. The arrows indicate the positions of the genes and the directions of transcription. Putative transcriptional terminators (circled *t*'s) are indicated. The proteins encoded by the genes are as follows: *phhR*, the σ^{54} transcriptional activator of the *phh* operon (28); *phhA*, phenylalanine hydroxylase; *phhB*, 4a-carbinolamine dehydratase; and *phhC*, aromatic aminotransferase (8). (B) Regeneration of the pterin cofactor for phenylalanine hydroxylase. The enzymes involved are as follows: PhhA, phenylalanine hydroxylase; PhhB, 4a-carbinolamine dehydratase; and DHPR, dihydropteridine reductase.

dimensional structures for mammalian DCoH (6) and *P. aeruginosa* PhhB (7) have been resolved, revealing superimposable three-dimensional structures. Each contains a saddle-shaped groove which is believed to comprise a probable macromolecule-binding site. The structure also manifests a protein motif reminiscent of the molecular saddle seen in the TATA binding protein (19). However, DCoH affinity for DNA or RNA has not been observed (16, 23). The relationship of the conserved structural features to the regulation mechanism operating in organisms as diverse as humans and bacteria is a very intriguing question. In this context of broad significance, we examined the regulatory properties of PhhB in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains, plasmids, phages, and media. The bacterial strains, plasmids, and phages used in this study are listed in Table 1. The Luria-Bertani (LB) and M9 formulations (24) were used as enriched and minimal growth media, respectively. *Pseudomonas* isolation agar (Difco) was used for isolating *P. aeruginosa* knockout mutants. Additions of ampicillin (100 μg/ml), chloramphenicol (40 μg/ml), kanamycin (50 μg/ml), mercury chloride (15 μg/ml), phenylalanine (50 μg/ml), and thiamine (17 μg/ml) were made as appropriate. Agar (Difco) was added at a final concentration of 2% (wt/vol) for preparation of solid medium.

Recombinant DNA techniques. Molecular cloning and DNA manipulation, including plasmid purification, restriction enzyme digestion, ligation, and transformation, were conducted by standard methods (24). DNA fragments were purified from agarose gel with a GeneClean kit (Bio 101). Electroporation (Invitrogen) was used for simultaneous transformation of *E. coli* with two compatible plasmids. Restriction enzymes, T4 DNA ligase, DNA-modifying enzymes (New England Biolabs or Promega), *Taq* DNA polymerase (Perkin-Elmer), and Vent DNA polymerase (New England Biolabs) were used as recommended by the suppliers.

Phenylalanine hydroxylase assay. *E. coli* JP2255 harboring pJZ9-3a was grown at 37°C in 500 ml of LB broth supplemented with ampicillin and harvested at the late-exponential phase of growth. Cell pellets were resuspended in 8 ml of 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol and were disrupted by sonication. The resulting extract was centrifuged at 150,000 × *g* for 1 h at 4°C. The supernatant was desalted with Sephadex G-25 and used as crude extract for the enzyme assay. PhhA activity was assayed by monitoring tyrosine formation (18).

Assay for PhhB activity. PhhB activity in *E. coli* (pJZ9-4a) was assayed either indirectly by the phenylalanine hydroxylase stimulation assay (11, 15) or directly with a more straightforward assay (22).

Construction of *phhA-lacZ* transcriptional fusions. For the transcriptional fusion (*phhA′-lacZ*), the *Hin*cII-*Bam*HI fragment containing the upstream region of *phhA* was first cloned into pACYC177 (which had been digested with *Hin*cII and *Bam*HI) to create pJS51. A *Bam*HI cassette of a promoterless *lacZ* gene from Z1918 was then inserted at the *Bam*HI site of pJS51 in the same orientation as the *phhA* gene to create pJS51Z, which was used as a multicopy *phhA′-lacZ* fusion. A single-copy *phhA′-lacZ* fusion was obtained by converting pJZ51Z into λ(*phhA′-lacZ*) by following the procedure described by Yu and Reznikoff (33).

Construction of *phhA-lacZ* translational fusions. For the translational fusion (*phhA′-′lacZ*), the *Hin*cII-*Bam*HI fragment containing the upstream region of the *phhA* gene was generated by PCR with the upstream primer 5′-GACAGA GCAGGTAGATGGCGTT-3′ and the downstream primer 5′-GGGATCCGGC TCGTGGGGCAGGCCGA-3′ (*Bam*HI site underlined). An extra guanine nucleotide (in boldface type) was added in the downstream primer to create the frameshift needed for an in-frame fusion at the *Bam*HI site to generate *phhA′-′lacZ*. The *Hin*cII-*Bam*HI fragment with the frameshift was inserted into the *Hin*cII-*Bam*HI site of pACYC177 to create pJS105, and a *Bam*HI cassette of truncated *′lacZ* from pMC1871 was inserted into pJS105 to create the translational fusion plasmid pJS105Z.

β-Galactosidase assay. β-Galactosidase activity was assayed under conditions of proportionality as described by Miller (17), and specific activities are ex-

TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Genotype or description	Source or reference
Strains		
E. coli		
BL21(DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm*; with DE3, a λ prophage carrying the T7 RNA polymerase gene	Novagen
DH5α	F^- Δ*lacU169 f80 dlacZ*ΔM15 *hsdR17 recA1 endA1 gyrA96 thi-1 relA1 supE44*	GIBCO/BRL
LE392	F^- *el4* mutant ($McrA^-$) *hsdR514* ($r_K^- m_K^+$) *supE44 supF58 lacY1* or Δ(*lacIZY*)*6 galK galT22 metB1 trpR55*	24
JP2255	*aroF363 pheA361 pheO352 tyrA382 thi-1 strR712 lacY1 xyl-15*	1
S17-1	[RP4-2 (Tc::Mu) (Km::Tn*7*) Tra (IncP)] *pro hsdR recA* Tp^r Sm^r	27
JS1	SP1313F(*phhA′-lacZ*)	This study
SP1313	*zah-735*::Tn*10*Δ(*argF-lac*)*U169* Δ(*tyrR*)	9
P. aeruginosa		
PA01	Prototroph	10
JS101	PA01 *phhA* Hg^r	28
JS102	PA01 *phhR* Hg^r	28
JS103	PA01 *phhB* Hg^r	This study
JS104	PA01 *phhC* Hg^r	8
Plasmids		
pUC18	Amp^r *lac′IPOZ′*	32
pUC19	Amp^r *lac′IPOZ′*	32
pACYC177	P15A replicon, Ap^r Km^r	2
pET11b	T7 *lac* promoter *lacI*$^+$ Ap^r	Novagen
pET23	T7 *lac* promoter, *lacI*$^+$ Ap^r	Novagen
pGEM-3Z	T7 promoter, Ap^r	Pomega
pGST-DCoH	In-frame protein fusion of glutathione *S*-transferase and DCoH	3
pJS10	*phhAB*, 2.5-kb *Hin*cII fragment cloned into pGEM-3Z behind the T7 promoter	This study
pJS11	*phhAB′*, 1.44-kb *Hin*cII-*Eco*RV fragment cloned into pACYC177	This study
pJS12	*phhAB*, 2.5-kb *Hin*cII fragment cloned into *Hin*cII site of pACYC177	This study
pJS51	*Hin*cII-*Bam*HI fragment containing truncated *phhA′* cloned into pACYC177	This study
pJS51Z	*phhA′-lacZ* transcriptional fusion in pACYC177	This study
pJS63	*phh′ABC*, *Bam*HI-*Hin*dIII fragment cloned into pGEM-3Z behind the T7 promoter	This study
pJS72	*phhA*, PCR-generated fragment containing the native RBS and PhhA-coding region cloned into pET23 behind the T7 *lac* promoter	This study
pJS95	PhhA overexpression vector; PhhA-coding region fused with T7 translational initiation signal at *Nde*I site of pET11b	This study
pJS96	PhhA overexpression vector; *phhA* fused with the T7 translational initiation signal cloned into pUC19 behind *lac* promoter to constitutively overexpress PhhA	This study
pJS97	PhhA overexpression vector; *phhA* fused to the T7 translational signal cloned into pTrc99A behind the *trc* promoter	This study
pJS101	Hg^r cassette, Ap^r	28
pJS105	*Hin*cII-*Bam*HI PCR fragment containing *phhA′* with a frameshift	This study
pJS105Z	*phhA′-′lacZ* protein fusion cloned into pACYC177	This study
pJZ9	*phhRABC* Ap^r	34
pJZ9-3a	*phhAB* Ap^r	34
pJZ9-4	*phh′ABC* Ap^r	34
pJZ9-5	*phhAB′* Ap^r	34
pMC1871	*lacZ* protein fusion vector	Pharmacia
pTrc99A	*trc* promoter, *lacI*$^+$ Ap^r	Pharmacia
pUFR004	ColE1 replicon, Cm^r Mob^+ *mob*P *lacZa*$^+$	5
Z1918	Promoterless *lacZ*, Ap^r	26
Phages		
λRZ5	λ *′bla ′lacZ lacY*$^+$	21
λJS1	λφ(*phhA′-lacZ*) *lacY*$^+$ *′bla*	This study

pressed in Miller units. The data are the results of at least two independent assays.

Construction of PhhA and PhhB overexpression vectors. To express PhhA protein at high levels, the overexpression plasmids pJS72 and pJS95 were constructed. A PCR fragment containing the complete coding region of *phhA* and the native ribosome-binding site (RBS) was amplified by use of the upstream primer 5′-CATGGAGTCCGT**ATG**AAAACGACGCA-3′ (RBS underlined; ATG start codon in boldface type) and the downstream primer 5′-CTTGGTT GTCGCATGTGGGAGCGGCG-3′ and cloned into pET23 behind the T7 *lac* promoter to create pJS72. pJS95 was constructed by inserting the coding region of *phhA* into the translational fusion vector pET11b. The coding region was amplified by PCR with the upstream primer 5′-CCAT**ATG**AAAACGACGCA GTACGTG-3′ and the downstream primer 5′-CAAGTCTGGTTGTCGCATG TGGGAGCGGCG-3′. The upstream primer was made with a built-in *Nde*I site (underlined), allowing fusion of *phhA* at the translational start site (ATG in boldface type) with the T7 translational initiation signals. To overexpress the PhhA protein constitutively, the *phhA*-coding region together with the upstream T7 translational start signals was excised from pJS95 as an *Xba*I fragment and cloned into pUC18 downstream of a *lac* promoter to create pJS96. The *Xba*I fragment was also cloned into pTrc99A downstream of the inducible *trc* promoter to create pJS97.

Two similar plasmids, pJS10 and pJS63, were constructed to overexpress

PhhB. The *Hinc*II fragment containing both the *phhA* and the *phhB* gene was inserted into pGEM-3Z to create pJS10, and the *Bam*HI-*Hin*dIII fragment containing both *phhB* and *phhC* was inserted into pGEM-3Z to create pJS63. In both plasmids, the *phhB* gene was under the control of a T7 promoter.

SDS-PAGE and Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide) was performed with a Mini-PROTEAN II cell (Bio-Rad) by the method of Laemmli (14). Samples of exponential-phase cells were collected by centrifugation, and the cell pellets were suspended in gel loading buffer and heated at 100°C for 10 min. Samples of 5 to 10 μl were loaded onto two SDS-polyacrylamide gels. After separation of the proteins by electrophoresis, one gel was stained with Coomassie blue and the other gel was used for blotting. When crude extracts were used, equivalent amounts of protein were loaded into each lane. Western blots were performed according to the method of Towbin et al. (30). The proteins were electrophoretically transferred onto nitrocellulose membranes and reacted with the polyclonal antibodies raised against PhhA or PhhB in a rabbit. Quantitative estimates were made by densitometry.

Gene inactivation. The *PhhB* gene was inactivated by following the method described by Song and Jensen (28). To generate the truncated *′phhB′* fragment (308 bp), the upstream primer 5′-ACCCAAGCCCATTGCGAAGCCTGCC G-3′ and the downstream primer 5′-GTGCGCGCCGCCATGATGAAATCGT T-3′ were used. Interruption of the *phhB* gene in an Hg^r isolate was confirmed by Southern hybridization.

Immunoaffinity chromatography and immunoprecipitation assays for protein complex. For immunoaffinity chromatography, anti-PhhB antibody was cross-linked to CNBr-activated Sepharose 4B which was packed into a 10-ml column and used according to the protocol described by the supplier (Pharmacia). The anti-PhhB affinity column was preequilibrated with buffer A (0.1 M potassium phosphate buffer [pH 7.4], 0.25 M NaCl, 1 mM dithiothreitol, 10 mM Fe^{2+}). Crude extract of *E. coli* JP2255 harboring pJZ9-3a was applied to the anti-PhhB affinity column. After being washed extensively with buffer A (over 20 bed volumes), the column was eluted with 0.1 M glycine-HCl buffer (pH 2.8) and the collected fractions were analyzed for coelution of PhhA and PhhB by Western blotting.

Coimmunoprecipitation of PhhA and PhhB. A crude extract prepared from *E. coli* JP2255 harboring pJZ9-3a was used as the enzyme source. The extract was mixed with anti-PhhA, anti-PhhB, or preimmune (control) serum in a ratio of 1:1 (vol/vol) and incubated at 25°C for 10 min just before the assays. The assays for phenylalanine hydroxylase activity in the antiserum-treated preparations were carried out as described above.

Biochemicals and molecular biology reagents. Dihydropteridine reductase and 6,7-dimethyl-5,6,7,8-tetrahydropteridine were obtained from Sigma (St. Louis, Mo.). Rat liver phenylalanine hydroxylase was prepared by following a published protocol (13). Antisera to PhhA and PhhB were produced in rabbits (Cocalico Biologicals, Inc., Reamstown, Pa.). Restriction enzymes, T4 DNA ligase, DNA-modifying enzymes (New England Biolabs or Promega), and *Taq* DNA polymerase (Perkin-Elmer) were used as recommended by the suppliers. Other biochemical agents were purchased from Sigma or Aldrich. Inorganic chemicals (analytical grade) were from Fisher Scientific.

RESULTS

***Trans*-complementation of tyrosine auxotrophy by *phhA* and *phhB*.** Both *phhA* and *phhB* had been shown to be needed for functional complementation of tyrosine auxotrophs in *E. coli* (34), but it was unknown whether the requirement for *phhB* was *cis* or *trans* with respect to *phhA*. A *trans*-complementation study was done, and this ruled out a *cis* effect of *phhB* on the expression of *phhA*. *phhA* and *phhB* (or *DCoH*) were cloned into two compatible plasmids, pJS11 and pJZ9-4 (or pGST-DCoH), respectively. The results showed that *phhB* was able to complement *E. coli* tyrosine auxotrophy in *trans* with respect to *phhA*. Furthermore, mammalian DCoH was able to replace PhhB in the bacterial system.

Effect of *phhB* knockout in *P. aeruginosa*. The *phhB* gene in *P. aeruginosa* was inactivated by interrupting the chromosomal *phhB* gene with insertion of the suicide plasmid pUFR/*′phhB′*/Hg^r through a single homologous crossover event (28). The interruption of the *phhB* gene in the resulting mutant was confirmed by Southern blot analysis (data not shown).

To determine the effect of *phhB* knockout, we used Western blots to monitor the expression of PhhA and PhhB in the *phhB* mutant, along with other gene knockout strains (Fig. 2A). The PhhA level in the *phhB* mutant, compared to that of the wild-type strain, was reduced by about 2.5-fold. It was unchanged in a *phhC* knockout strain. PhhA was not detected in the *phhA* knockout mutant (negative control). The PhhA level was significantly reduced in the *phhR* knockout strain, as we previously reported (28). The absence of PhhB in the PhhA knockout strain is presumably due to the polar effect of the insertion.

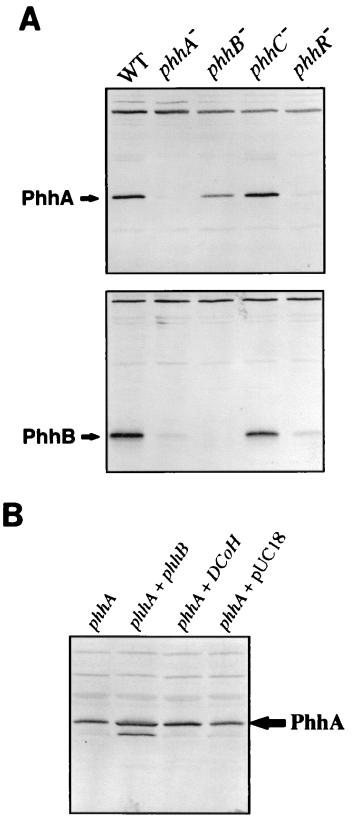

FIG. 2. Activation of *phhA* expression by PhhB. (A) Western blot analysis of PhhA (top blot) and PhhB (bottom blot) levels in *P. aeruginosa* knockout mutants. Lanes from left to right were loaded with lysate preparations from the wild-type strain PA01 (WT) and its knockout mutant derivatives with gene interruptions in *phhA*, *phhB*, *phhC*, or *phhR*. Proteins in whole-cell lysates were separated by SDS-PAGE and probed with rabbit anti-PhhA or anti-PhhB polyclonal antibodies. (B) Western blot analysis of PhhA expression in *E. coli* JP2255. Proteins in the whole-cell lysates of JP2255 carrying the plasmids indicated below were separated by SDS-PAGE and reacted with rabbit anti-PhhA polyclonal antibodies. Plasmids containing the gene(s) indicated at the top of the gel were as follows: *phhA*, pJS11; *phhB*, pJZ9-4; and DCoH, pGST-DCoH. PUC18 was used as the control plasmid. The lower band in lane 2 is probably a fusion protein from pJZ9-4 which contains a fragment of *phhA* fused to the *lacZ* alpha fragment, which is recognized by the polyclonal anti-PhhA antibodies.

The physiological effect of the *phhB* knockout in *P. aeruginosa* was also examined. Inactivation of *phhB* abolished its ability to grow on either phenylalanine or tyrosine as the sole carbon source. However, since *phhC* has been found to be essential for growth on either phenylalanine or tyrosine as the sole carbon source (8), we do not know the extent to which this can be attributed to a polar effect of the insertion on downstream *phhC*.

PhhB regulates expression at the posttranscriptional level. To determine whether the regulation of *phhA* expression by PhhB is exerted at the transcriptional or translational level, we constructed both transcriptional and translational fusions using *lacZ* as a reporter gene. For the transcriptional fusion, we constructed *phhA′-lacZ* fusions in both a multicopy form (pJS51Z) and a single-copy from [λ(*phhA′-lacZ*)]. In both cases, addition of PhhB (pJZ9-4) or DCoH (pGST-DCoH) did not result in a level of β-galactosidase higher than that in the

TABLE 2. Effect of *phhB* or *DCoH* supplied in *trans* upon expression of *phhA* in transcriptional or translational fusions[a]

Plasmid used in *trans*	Gene supplied in *trans*	β-Galactosidase activity[b]		
		pJS51Z	λJS1	pJS105Z
pJZ9-4	*phhB*	180.7 ± 40.6	13.2 ± 1.2	13.1 ± 0.1[c]
pGST-DCoH	*DCoH*	190.6 ± 13.1	14.1 ± 0.8	10.4 ± 0.3[c]
pUC18	None	182.3 ± 16.3	15.7 ± 1.2	7.3 ± 0.7

[a] Regulation of *phhA* expression was studied with *lacZ* as the reporter gene. β-Galactosidase activities in the transcriptional fusions pJS51Z (*phhA'-lacZ*) (multicopy) and λJS1 (*phhA'-lacZ*) (single copy) and the translational fusion pJS105Z (*phhA'-'lacZ*) (multicopy) were assayed in the absence and presence of either PhhB or DCoH.

[b] β-Galactosidase activities are reported in Miller units. Values are means (± standard deviations) of results from three independent experiments.

[c] Significantly higher than the value for the control (pUC18) ($P < 0.0001$ by analysis of variance).

control (pUC18) (Table 2), indicating that neither PhhB nor DCoH produced any effects at the transcriptional level.

We also used a translational fusion *phhA'-'lacZ* (pJS105Z) (Table 2), and PhhB or DCoH was again provided in *trans* on a second plasmid. The results showed about a twofold increase in β-galactosidase activity, indicating that PhhB regulates the expression of *phhA* at a posttranscriptional level. Activation by PhhB measured in this way is consistent with the results obtained from Western blot analysis for both *P. aeruginosa* (Fig. 2A) and *E. coli* (Fig. 2B), where similarly modest levels of activation in *phhA* expression were observed.

PhhA protein is expressed in the absence of PhhB. We expected the *phhB* knockout result with *P. aeruginosa* and the gene fusion experiments with *E. coli* (Table 2) to show much greater effects of PhhB's absence than the relatively modest reductions in levels of PhhA that were observed. This was because in previous work (34), little or no PhhA had been detected in the absence of *phhB* (pJZ9-5) in *E. coli* whereas a very high level of PhhA was produced in the presence of *phhB* (pJZ9-3a). Therefore, we reevaluated the ability of PhhB to enhance the expression of *phhA* in *E. coli* JP2255 (Fig. 2B). A substantial level of PhhA was, in fact, detected when only *phhA* was present. A further twofold increase in PhhA level was found when either *phhB* or *DCoH* was also present. The latter results are consistent with the foregoing results with the *P. aeruginosa phhB* knockout mutant (Fig. 2A) and with the translational fusion results with *E. coli* (Table 2). The inconsistency between the result obtained with pJS11 (Fig. 2B, lane 2) and the result reported for pJZ9-5 (34) was found to be due to the incorrect orientation of the insert in pJZ9-5. Partial sequencing of pJZ9-5 revealed that the *phhA* gene was positioned opposite to the *lac* promoter, rather than in the same orientation as had been claimed.

Expression of PhhA without PhhB has growth-inhibitory effects in *E. coli*. Since it is now clear that PhhA can be expressed in the absence of PhhB, the question arises as to why *phhB* is needed in combination with *phhA* for functional complementation. The most obvious consideration is whether blockage of pterin recycling stops the phenylalanine hydroxylase reaction in vivo. Although dihydropteridine reductase, one of two essential enzymes in recycling of the pterin cofactor, has been found in *E. coli* (31), 4a-carbinolamine dehydratase activity has not been detected in this bacterium and there is no ortholog of *phhB* present in the genome. Nevertheless, the absolute requirement for 4a-carbinolamine dehydratase activity to obtain complementation is perhaps surprising because this reaction proceeds to some extent nonenzymatically. It is possible that excessive utilization of an *E. coli* pterin in the absence of PhhB causes some nutritional limitation. We considered the possibility that tetrahydrofolate biosynthesis is affected and hence reduces the pool of methyl group donors. However, supplementation of DH5α/pJS96 on LB broth plates with L-methionine or with *S*-adenosylmethionine failed to relieve inhibition.

Another possibility for the requirement of 4a-carbinolamine dehydratase in complementation is its use to overcome some mechanism of toxicity, perhaps caused by an accumulated carbinolamine derivative such as 7-biopterin. Growth inhibition by PhhA was indeed demonstrated by comparison of two constructs having different basal levels of PhhA expression (Fig. 3A). In one construct the *Xba*I fragment from pJS95 carrying *phhA* (fused with ϕ10 translational signals) was cloned into pTrc99A behind the inducible *trc* promoter to create pJS97. pJS97 carries *lacI*^{q}, and *E. coli* DH5α carrying pJS97 was plated on LB broth-ampicillin plates with different concentrations of IPTG (isopropyl-β-D-thiogalactopyranoside; 1 to 5 mM). A control plate with no IPTG added was also used. Pinpoint colonies emerged on the control plate after overnight incubation at 37°C, while 2 days was required to see pinpoint colonies on IPTG-containing plates. Progressively higher concentrations of IPTG correlated with greater elapsed times before visible colonies appeared. Thus, it appears that even the low basal output of PhhA from the strong *trc* promoter is sufficient to trigger growth inhibition. When *E. coli* DH5α carrying pJS96 (a plasmid construct where *phhA* is constitutively overexpressed from the *lac* promoter) was used, an elapsed time of several days was required to see pinpoint colonies. Both constructs, especially pJS96, are unstable and are readily lost from host cells. Given its relative lack of promoter leakiness, we were able to overexpress PhhA from pJS97 (Fig. 3B) upon IPTG induction. Because PhhA is constitutively overexpressed and because of the consequent selective pressure for a high rate of plasmid loss, we were not able to produce a high level of PhhA from pJS96 (Fig. 3B). Neither pJS96 nor pJS97 alone was able to complement *E. coli* tyrosine auxotrophy; however, they were able to complement the auxotrophy with the additional provision of *phhB* in *trans* on pJZ9-4 (data not shown).

Coexpression of PhhA and PhhB in *P. aeruginosa*. Expression of both *phhA* and *phhB* was induced by the presence of phenylalanine in *P. aeruginosa* grown on a minimal fructose-based medium (Fig. 4). Coinduction of *phhA* and *phhB* were expected, since they coexist in the *phh* operon. However, a basal level of PhhB was expressed under noninducing conditions as seen in Fig. 4, lane 2, on the Western blot, whereas PhhA was not detected. This result suggests the existence of default expression of *phhB* from a weak, internal promoter under noninducing conditions.

PhhA and PhhB form a complex. We noticed that when crude extracts of *E. coli*(pJZ9-3a), which possesses both PhhA and PhhB, were treated with antibody to PhhB, effective immunoprecipitation of PhhA occurred (Table 3). Since the anti-PhhB antibody does not affect PhhA alone, we conclude that PhhA and PhhB can exist as a dissociable complex. Anti-PhhB antibody presumably precipitates PhhA indirectly by virtue of the protein-protein association of PhhA and PhhB. Consistent with this conclusion were the results of affinity chromatography in which anti-PhhB antibody was immobilized on an affinity column (see Materials and Methods). Passage of extract containing both PhhA and PhhB through the column resulted in retention of both PhhA and PhhB.

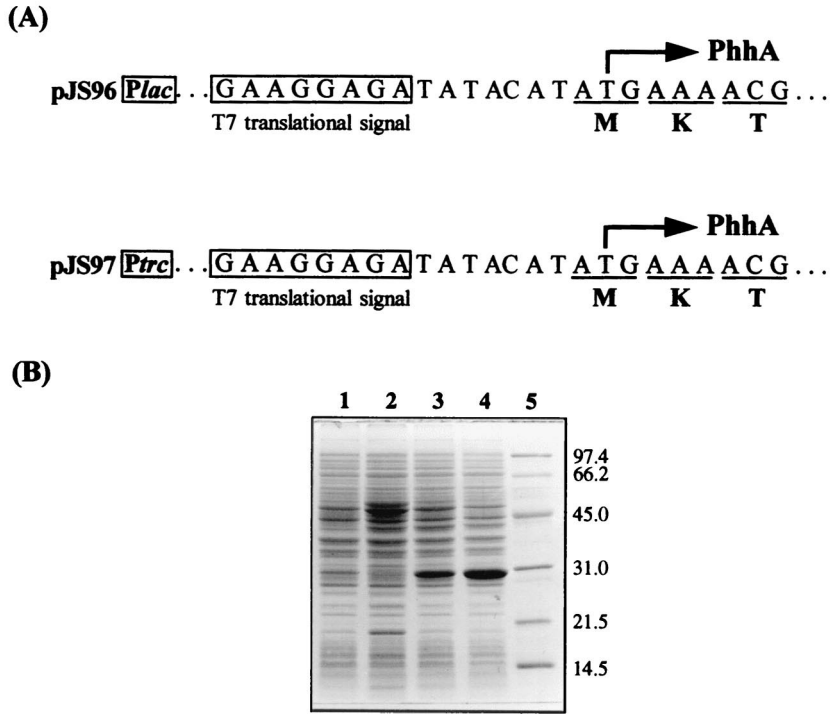

FIG. 3. Expression of PhhA in *E. coli* JP2255. (A) Construction of the PhhA expression plasmids pJS96 and pJS97. (B) SDS-PAGE analysis of PhhA expression. Proteins in whole-cell lysates were separated by SDS-PAGE and stained with Coomassie blue. Lane 1, JP2255/pJS96; lane 2, JP22255/pTrc99A (control); lanes 3 and 4, JP2255/pJS97 before and after induction with 1 mM IPTG for 3 h at 30°C, respectively; lane 5, molecular weight markers (in thousands).

DISCUSSION

Phenylalanine hydroxylase in nature. Phenylalanine hydroxylase systems appear to be of infrequent occurrence in prokaryotes. Phenylalanine hydroxylase has been reported in a few species belonging to the α division of the class *Proteobacteria* and is so far restricted to *P. aeruginosa* in the γ division (reference 34 and references therein). Of these, *P. aeruginosa* is the best characterized at the molecular-genetic level (28, 34), and the *phh* operon of *P. aeruginosa* is thus far unique. At this time homologs of pterin carbinolamine dehydratase encoded by *phhB* have not been reported for any other prokaryote. With the completion of the *P. aeruginosa* genome pending, it will be interesting to examine the exact enzymes of pterin biosynthesis that are present. Elucidation of the exact pathway of tyrosine catabolism used in *P. aeruginosa* is also forthcoming.

Toxicity triggered by PhhA expression. Mammalian defects in either of the enzymes required for pterin cofactor recycling, pterin carbinolamine dehydratase and dihydropteridine reductase, cause malignant hyperphenylalaninemia. *E. coli* tyrosine auxotrophs cannot be complemented by *P. aeruginosa phhA* in the absence of *phhB* (34). This result was interpreted to be the consequence of a positive regulatory effect of *phhB* upon *phhA* expression since no PhhA enzyme activity was measured (34). However, the construct used before was found to have an orientation opposite to that reported. The correct construct yields cell populations which do express PhhA in the absence of PhhB, albeit at reduced levels. What then accounts for the inability of plasmid constructs containing *phhA* alone to complement tyrosine auxotrophy in *E. coli*? One obvious possibility is that *phhB* is simply necessary in vivo in order to maintain pterin recycling, to ensure the availability of a reduced pterin to PhhA. However, one might have expected at least to obtain slow-growing tyrosine-independent transformants due to non-

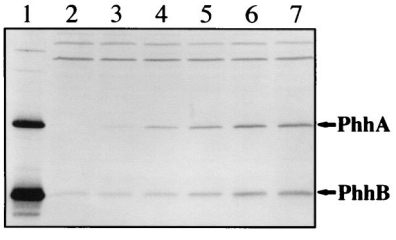

FIG. 4. Induction of the *phh* operon by phenylalanine in *P. aeruginosa*. Proteins in the whole-cell lysates were separated by SDS-PAGE (12%). Lane 1, *E. coli* JP2255 harboring both pJS11 and pJZ9-4; lanes 2 to 7, samples taken from wild-type *P. aeruginosa* PA01 grown on M9 minimal medium after elapsed times of 0, 10, 30, 60, 90, and 120 min, respectively, following addition of 100 μg of L-phenylalanine per ml at zero time.

TABLE 3. Immunoprecipitation of PhhA activity by anti-PhhB antibody in the presence of PhhB[a]

Antibody[b]	Sp act[c]	Relative activity[d]
Preimmune serum	54.8 ± 1.9	100
Anti-PhhA serum	12.2 ± 0.6*	22.3
Anti-PhhB serum	14.9 ± 0.6*	27.3

[a] *E. coli* (pJZ9-3a) crude extract containing both PhhA and PhhB was used as the enzyme source in the assay.

[b] Rabbit antisera against PhhA and PhhB and preimmune serum (control) were used.

[c] The specific activity of phenylalanine hydroxylase is defined as nanomoles of tyrosine formed per minute per milligram of protein and is expressed as a mean value ± the standard deviation. *, significantly different from the value for the preimmune control ($P < 0.001$).

[d] Relative activity is expressed as a percentage of the activity of the control (preimmune serum).

enzymatic dehydration. A second possibility to account for the inability of PhhA to function in vivo in the absence of PhhB is toxicity caused by PhhA. Indeed, we found strong growth inhibition in *E. coli* strains in which *phhA* was expressed in the absence of *phhB* under growth conditions where PhhA conferred no selective value. 7-Biopterin is nonenzymatically produced from the pterin product formed (4a-hydroxytetrahydrobiopterin) after utilization of BH_4 during PhhA catalysis in the absence of carbinolamine dehydratase, and 7-biopterin may be toxic. Perhaps it interferes with some pterin-dependent system of *E. coli*.

It is quite possible that *E. coli* (and *P. aeruginosa*) provides PhhA with a functional pterin cofactor other than BH_4. The complete *E. coli* genome contains genes for GTP cyclohydrolase I (EC 3.5.4.16) and 6-pyruvoyltetrahydropterin synthase (EC 4.6.1.10). Sepiapterin reductase, which can accomplish the two-step conversion of 6-pyruvoyltetrahydropterin to BH_4, appears to be absent. Perhaps another reductase which is the functional equivalent of sepiapterin reductase is present. The most parsimonious explanation is that 6-pyruvoyltetrahydropterin is the pterin moiety utilized in vivo, but its lability seemingly rules this out. Another possibility is utilization of tetrahydroneopterin by PhhA and the ability of dihydropteridine reductase to recognize dihydroneopterin (which is present in *E. coli*). If so, growth inhibition in the presence of *P. aeruginosa phhA* may be due to the carbinolamine derivative generated from tetrahydroneopterin during phhA catalysis, an effect rescued by the carbinolamine dehydratase activity of PhhB.

Regulatory relationships between *phhA* and *phhB*. The *phh* operon contains the three structural genes *phhA*, *phhB*, and *phhC* and the divergently transcribed, positively acting regulatory gene *phhR*, as illustrated in Fig. 1. *phhA* and *phhB* have been shown to be subject to coordinate induction when either phenylalanine or tyrosine is present in the growth medium. Levels of PhhC could not be monitored due to lack of specific antibody and the presence of other aminotransferase species with overlapping substrate specificities. Since *phhC* and *phhB* are translationally coupled, it is likely that *phhC* and *phhB* are expressed coordinately. Under growth conditions where induction is not promoted by addition of aromatic amino acids, the expression of *phhA* and that of *phhB* are not coordinate. PhhA cannot be detected, whereas PhhB maintains a distinct basal level of activity, which suggests that a weak internal promoter may lie between *phhA* and *phhB* to ensure basal levels of PhhB (and PhhC).

PhhB exerts a positive regulatory effect upon levels of expression of PhhA, an effect which was consistently two- to threefold in magnitude. Since the quantitative effect of the presence of PhhB in *E. coli* was the same as in *P. aeruginosa*, the positive regulatory effect of *phhB* must be independent of *phhR* (because *E. coli* lacks *phhR*). We found that PhhB regulates *phhA* at the posttranscriptional level. This result is contrary to results reported elsewhere, but no data were given (16). Among the possible mechanisms of activation are that (i) PhhB may bind to *phhA* mRNA and either enhance translational initiation or protect the mRNA from degradation and (ii) PhhB may directly bind to PhhA and promote some catalytic or stability enhancement. Consistent with the latter possibility are results which indicate that PhhA and PhhB form a dissociable protein-protein complex.

ACKNOWLEDGMENTS

We thank S. Kaufman for providing purified rat liver phenylalanine hydroxylase and J. Ayling for suggesting the possible alternative pterin recycling mechanisms which might be employed by *E. coli*.

REFERENCES

1. **Baldwin, G. S., and B. E. Davidson.** 1981. A kinetic and structural comparison of chorismate mutase/prephenate dehydratase from mutant strains of *Escherichia coli* K-12 defective in the *pheA* gene. Arch. Biochem. Biophys. **211:**66–75.
2. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
3. **Citron, B. A., M. D. Davis, S. Milstien, J. Gutierrez, D. B. Mendel, G. R. Crabtree, and S. Kaufman.** 1992. Identity of 4**a**-carbinolamine dehydratase, a component of the phenylalanine hydroxylation system, and DCoH, a transregulator of homeodomain proteins. Proc. Natl. Acad. Sci. USA **89:**11891–11894.
4. **Curtius, H. C., C. Adler, I. Rebrin, C. Heizmann, and S. Ghisla.** 1990. 7-Substituted pterins: formation during phenylalanine hydroxylation in the absence of dehydratase. Biochem. Biophys. Res. Commun. **172:**1060–1066.
5. **DeFeyter, R., C. I. Kado, and D. W. Gabriel.** 1990. Small, stable shuttle vectors for use in *Xanthomonas*. Gene **88:**65–72.
6. **Endrizzi, J. A., J. F. Cronk, W. Wang, G. R. Crabtree, and T. Alber.** 1995. Crystal structure of DCoH, a bifunctional, protein-binding transcriptional coactivator. Science **268:**556–559.
7. **Ficner, R., U. H. Sauer, G. Stier, and D. Suck.** 1995. Three-dimensional structure of the bifunctional protein PCD/DCoH, a cytoplasmic enzyme interacting with transcriptional factor HNF1. EMBO J. **14:**2034–2042.
8. **Gu, W., J. Song, C. A. Bonner, G. Xie, and R. A. Jensen.** 1998. PhhC, an essential aminotransferase for aromatic amino acid catabolism in *Pseudomonas aeruginosa*. Microbiology **144:**3127–3134.
9. **Heatwole, V. M., and R. L. Somerville.** 1991. The tryptophan-specific permease gene, *mtr*, is differentially regulated by the tryptophan and tyrosine repressors in *Escherichia coli* K-12. J. Bacteriol. **173:**3601–3604.
10. **Holloway, B. W.** 1955. Genetic recombination in *Pseudomonas aeruginosa*. J. Gen. Microbiol. **13:**572–581.
11. **Huang, C. Y., E. E. Max, and S. Kaufman.** 1973. Purification and characterization of phenylalanine hydroxylase-stimulating protein from rat liver. J. Biol. Chem. **248:**4235–4241.
12. **Kaufman, S., B. A. Citron, M. Davis, and S. Milstien.** 1993. The isolation and characterization of clones of 4**a**-hydroxytetrahydrobiopterin dehydratase. Adv. Exp. Med. Biol. **338:**97–102.
13. **Kaufman, S., and D. B. Fisher.** 1970. Purification and some physical properties of phenylalanine hydroxylase from rat liver. J. Biol. Chem. **245:**4745–4750.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
15. **Lazarus, R. A., S. J. Benkovic, and S. Kaufman.** 1983. Phenylalanine hydroxylase stimulation protein is a 4a-carbinolamine dehydratase. J. Biol. Chem. **258:**10960–10962.
16. **Mendel, D. B., and G. R. Crabtree.** 1991. HNF1, a member of a novel class of dimerizing homeodomain proteins. J. Biol. Chem. **266:**677–680.
17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. **Nakata, H., T. Yamauchi, and H. Fujisawa.** 1979. Phenylalanine hydroxylase from *Chromobacterium violaceum*: purification and characterization. J. Biol. Chem. **266:**18454–18459.
19. **Nikolov, D. B., S. H. Hu, J. Lin, A. Gasch, A. Hoffmann, M. Horikoshi, N. H. Chua, R. G. Roeder, and S. K. Burley.** 1992. Crystal structure of TFIID TATA-box binding protein. Nature **360:**40–46.
20. **Pogge von Strandmann, E., and G. U. Ryffel.** 1995. Developmental expression of the maternal protein XDCoH, the dimerization cofactor of the homeoprotein LFB1 (HNF1). Development **121:**1217–1226.
21. **Rosentel, J. K., F. Healy, J. A. Maupin-Furlow, J. H. Lee, and K. T. Shanmugam.** 1995. Molybdate and regulation of *mod* (molybdate transport), *fdhF*, and *hyc* (formate hydrogenlyase) operons in *Escherichia coli*. J. Bacteriol. **177:**4857–4864.
22. **Rebrin, I., J. E. Ayling, J. Song, and R. A. Jensen.** 1997. Mechanistic studies of 4**a**-hydroxytetrahydropterin dehydratase from *Pseudomonas aeruginosa*, p. 631–634. *In* W. Pfleiderer and H. Rokas (ed.), Chemistry and biology of pteridines and folates. Blackwell Scientific, Oxford, United Kingdom.
23. **Rhee, K.-H., G. Stier, P. B. Becker, D. Suck, and R. Sandaltzopoulos.** 1997. The bifunctional protein DcoH modulates interactions of the homoeodomain transcription factor HNF1 with nucleic acids. J. Mol. Biol. **265:**20–29.
24. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
25. **Schallreuter, K. U., J. M. Wood, M. R. Pittelkow, M. Gutlich, K. R. Lemke, W. Rodl, N. N. Swanson, K. Hitzemann, and I. Ziegler.** 1994. Regulation of melanin biosynthesis in the human epidermis by tetahydrobiopterin. Science **263:**1444–1446.
26. **Schweizer, H. P.** 1993. Two plasmids, X1918 and Z1918, for easy recovery of the *xylE* and *lacZ* reporter genes. Gene **134:**89–91.
27. **Simons, R. W., F. Houman, and N. Kleckner.** 1983. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
28. **Song, J., and R. A. Jensen.** 1996. PhhR, a divergently transcribed activator of

the phenylalanine hydroxylase gene cluster of *Pseudomonas aeruginosa*. Mol. Microbiol. **22:**497–507.
29. **Thöny, B., F. Neuheiser, L. Kierat, M. Blaskovics, P. H. Arn, P. Ferreira, I. Rebrin, J. Ayling, and N. Blau.** 1998. Hyperphenylalaninemia with high levels of 7-biopterin is associated with mutations in the PCBD gene encoding the bifunctional protein pterin-**4a**-carbinolamine dehydratase and transcriptional coactivator (DcoH). Am. J. Hum. Genet. **62:**1302–1311.
30. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
31. **Vasudevan, S. G., D. C. Shaw, and W. L. F. Armarego.** 1988. Dihydropteridine reductase from *Escherichia coli*. Biochem. J. **255:**581–588.
32. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC vectors. Gene **33:**103–119.
33. **Yu, X.-M., and W. S. Reznikoff.** 1984. Deletion analysis of the CAP-cAMP binding site of the *Escherichia coli* lactose promoter. Nucleic Acids Res. **12:**5449–5464.
34. **Zhao, G., T. Xia, J. Song, and R. A. Jensen.** 1994. *Pseudomonas aeruginosa* possesses homologues of mammalian phenylalanine hydroxylase and 4**a**-carbinolamine dehydratase/DCoH as part of a three-component gene cluster. Proc. Natl. Acad. Sci. USA **91:**1366–1370.