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PhhR, a divergently transcribed activator of the phenylalanine hydroxylase gene cluster of *Pseudomonas aeruginosa*

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Summary

***Pseudomonas aeruginosa* was recently found to possess a cluster of structural genes encoding phenylalanine hydroxylase (PhhA), carbinolamine dehydratase (PhhB), and aromatic aminotransferase (PhhC). We now report the presence, in the flanking upstream region, of a divergently transcribed gene (*phhR*) encoding an activator protein. Inactivation of *phhR* markedly reduced expression of the structural genes. PhhR belongs to the large prokaryote family of σ^{54} enhancer-binding proteins, and activation of the *phh* operon by PhhR in *P. aeruginosa* required *rpoN*. The closest homologues of PhhR are the TyrR proteins from *Escherichia coli* and *Haemophilus influenzae*. *E. coli* TyrR is an unusual member of the homologue family in that the transcriptional units regulated by *tyrR* are driven by σ^{70} promoters. *P. aeruginosa phhR* was able to replace *E. coli tyrR* as a repressor of the *aroF–tyrA* operon (but not as an activator of *mtr*) in the heterologous *E. coli* system. Two regions that resemble *E. coli* TyrR boxes were identified in the intervening region between *phhR* and *phhA*. We propose that one or both boxes may be the target of PhhR acting as an autogenous repressor at a σ^{70} promoter in one direction. In the other direction, one or both boxes may be the upstream activator sequence targeted by PhhR to facilitate expression of the *phh* operon from a σ^{54} promoter. The *phh* operon was strongly induced in fructose- or glucose-based minimal medium by L-phenylalanine. Inactivation of *phhR* in *P. aeruginosa* abolished ability to utilize either L-phenylalanine or L-tyrosine as a sole source of carbon for growth.**

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Introduction

A recent report (Zhao *et al*., 1994) revealed that *Pseudomonas aeruginosa* possesses a tetrahydrobiopterin (BH_4)-dependent monooxygenase that is capable of catalysing the phenylalanine hydroxylase reaction. It is encoded by the proximal member (*phhA*) of a three-gene cluster. The second gene, *phhB*, encodes carbinolamine dehydratase, a key enzyme within the cycle regenerating BH_4. *phhC* encodes an aromatic aminotransferase and belongs to the large membership of Family I aminotransferases (Jensen and Gu, 1996). The reactions, as they are known to function for the mammalian homologues in the catabolism of L-phenylalanine, are shown in Fig. 1.

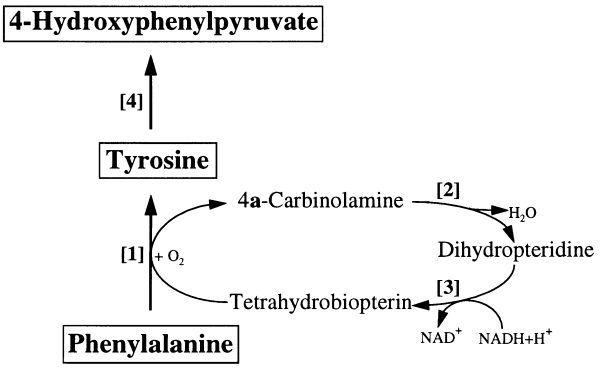
The physiological function of phenylalanine hydroxylase in *P. aeruginosa* has not been obvious. A primary role in L-tyrosine biosynthesis seems unlikely because of the established presence, for this purpose, of a cyclohexadienyl dehydrogenase that is widely distributed in Gram-negative bacteria and which is highly sensitive to feedback inhibition by L-tyrosine (Xia and Jensen, 1990). Although function as an initial step of L-phenylalanine catabolism has a precedent in mammalian metabolism, the literature encompassing the widely studied catabolism of aromatic compounds in pseudomonad bacteria (indeed, in prokaryotes) does not include the phenylalanine hydroxylase step. Furthermore, L-phenylalanine (substrate of PhhA) is an extremely poor source of carbon for growth of *P. aeruginosa*, whereas L-tyrosine (product of PhhA) is an excellent carbon source.

Zhao *et al*. (1994) had previously noted that subclones in *Escherichia coli* lacking the flanking regions around the *phh* operon possessed 20-fold greater activity for phenylalanine hydroxylase. This suggested to us the presence of a regulatory gene. Since an understanding of the regulation governing the *phh* operon should provide important physiological clues about function, we have analysed the flanking regions and now report the characteristics of a regulatory gene, denoted *phhR*.

Results

Evidence for a flanking regulatory region

The original clone (pJZ9) isolated by Zhao *et al*. (1994)



[1] Phenylalanine hydroxylase (**PhhA**)
[2] 4**a**-Carbinolamine dehydratase (**PhhB**)
[3] Dihydropteridine reductase
[4] Aromatic aminotransferase (**PhhC**)

Fig. 1. Initial reactions of phenylalanine catabolism in mammals. The three structural genes of the *phh* operon encode enzymes catalysing three of the four steps shown. 4**a**-Carbinolamine is an alternative designation for 4**a**-hydroxytetrahydrobiopterin.

produced markedly less phenylalanine hydroxylase activity than did subclone pJZ9-3a (Fig. 2A). A possible explanation was the presence of a negatively acting regulatory gene in either the upstream or downstream flanking region. pJS60, in correlation with its absence of upstream DNA but the presence of downstream DNA, expressed a very high level of activity. Thus, the upstream region appeared to be responsible for decreased expression of *phhA* in *E. coli*.

Transcriptional fusions were constructed using *lacZ* as a reporter gene, as shown in Fig. 2B. The results indicate that the negative effect conferred by upstream DNA occurs at the transcriptional level.

Identification of phhR

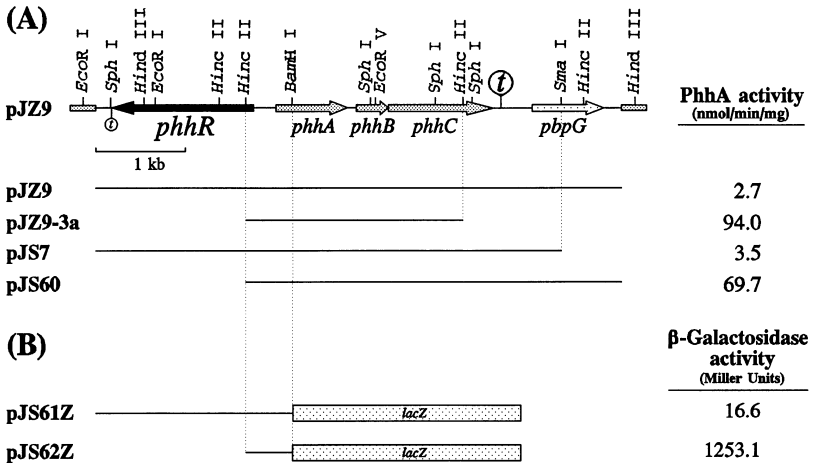
A large open reading frame (Fig. 2A) located upstream of the *phh* structural genes appeared likely to be functional according to the criterion of GCG codon-preference analysis. The gene, denoted *phhR*, produces a deduced protein having 518 residues, a molecular weight of 56 855, and an isoelectric point of 7.17. It contains only a single tryptophan residue. The open reading frame was confirmed by N-terminal amino acid sequencing (see the *Experimental procedures*).

Regions corresponding to a possible σ^{70} promoter region (TTGCTG-18-TATTCT) and a factor-independent transcription terminator (CGGCA-4-TGCCG) were found. A strong ribosome-binding site was not apparent. Perhaps the A-richness of the initiator region enhances ribosome binding (Ivey-Hoyle and Steege, 1992).

A physical map is given in Fig. 2A which shows the 5874 bp DNA segment containing the structural genes of the *phh* operon, the divergently transcribed regulatory gene *phhR*, and a gene (*pbpG*) downstream of the *phh* operon which encodes a penicillin-binding protein (J. Song and R. A. Jensen, unpublished).

Homology of PhhR with E. coli *TyrR*

The closest homologue of PhhR was found to be *E. coli* TyrR. The pairwise GAP alignment (GCG) is shown in Fig. 3. TyrR belongs to a family of modular proteins which usually have three functional domains. The alignment showed high amino acid identity throughout the entire lengths of PhhR and TyrR, and 45.7% of the deduced residues were identical. The N-terminal domain mediates regulatory modulations, and in TyrR these can involve any of the three aromatic amino acids. A central domain, highly conserved throughout the entire family of σ^{54} enhancer-binding proteins, exhibits two established motifs that have been shown in some members of the family to reflect the binding of ATP (Morrett and Segovia, 1993). Site A corresponds to the ATP-binding pocket motif and site B corresponds to segment 3 of adenylate



PhhA activity (nmol/min/mg)
2.7
94.0
3.5
69.7

β-Galactosidase activity (Miller Units)
16.6
1253.1

Fig. 2. A. Physical map of the DNA fragment containing *phh* structural genes, the divergently transcribed regulatory gene *phhR*, and the downstream penicillin-binding protein gene (*pbpG*) in the original clone pJZ9. Terminators downstream of *phhC* and *phhR* are indicated. The shaded bars at both ends are portions of the multiple cloning site of the pUC18 vector. The placement of restriction sites is shown. On the right phenylalanine hydroxylase (PhhA) activities are shown in *E. coli* JP2255 harbouring the different fragments shown on the left.
B. On the right, β-galactosidase activities are shown in BW545 harbouring the *phhA′–lacZ* transcriptional fusions diagrammed on the left.

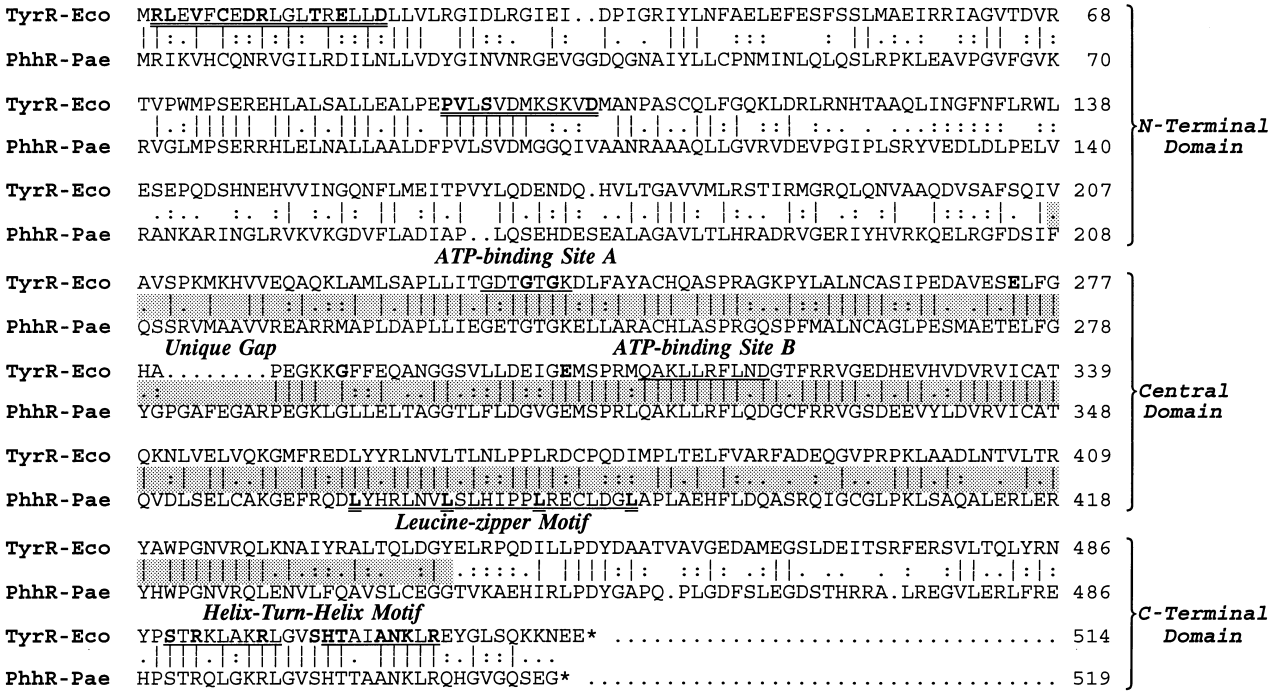


Fig. 3. Pairwise alignment (GAP program of GCG) of amino acid sequences corresponding to *P. aeruginosa* PhhR and *E. coli* TyrR. The similarity is 66.3% and identity is 45.7%. The three functional domains are indicated at the right, the central domain also being shaded. Domain boundaries are based upon those formulated by Morrett and Segovia (1993). Alternative boundaries based upon domain segments surviving partial hydrolysis by trypsin (Cui and Somerville, 1993) are residues 1–190, 191–467, and 468–513 for the three domains of Eco–TyrR, respectively. In the N-terminal domain, the region between amino acids 2 and 19 (which have a critical role in activation of the expression of *E. coli tyrP* and *mtr*) is double-underlined, and mutations at residues marked in bold abolish TyrR-mediated activation without affecting repression; a second region between amino acids 92 and 103 (which may play a subsidiary role in activation) is also double-underlined; mutations at the residues in bold resulted in loss of function (Pittard, 1996). In the central domain, two ATP-binding sites and a leucine-zipper motif are underlined. Mutations altering ATP-binding site A and mutations at the highly conserved residues E-274, G-285, and E-302 abolish TyrR-mediated repression. In the C-terminal domain, a helix-turn-helix DNA-binding motif is identified with the helix regions underlined and critical residues in bold print.

kinase. In this region a perfect leucine-zipper motif is apparent in *P. aeruginosa* PhhR, whereas *E. coli* displays an imperfect motif. Residues E-274, G-285, and E-302 were found to be important for TyrR-mediated repression of *aroF–tyrA* in *E. coli* (Yang *et al*., 1993; Kwok *et al*., 1995), and these residues are all conserved in *P. aeruginosa* PhhR. The C-terminal domain possesses a helix-turn-helix motif which is responsible for DNA binding (Yang *et al*., 1993). The absolute conservation of residues shown to be critical for DNA binding in *E. coli* (Pittard, 1996) strongly indicates that PhhR and TyrR might target a common DNA sequence.

In a situation similar to that of *E. coli* TyrR, the two aspartate residues and the lysine residue conserved in the amino-terminal domain of all response regulator proteins such as NtrC (Stock *et al*., 1989) were not found.

Overproduction of PhhR

PhhR protein was overexpressed in *E. coli* BL21(DE3) (as detailed in the *Experimental procedures*) by use of the T7 overexpression system; the construct is illustrated in Fig. 4. The initial use of overexpression vectors containing *phhR* on the *Bam*HI–*Sph*I fragment (see Fig. 2A) of pJZ9 failed. This is probably due to autogenous regulation of *phhR*, judging from the precedent set by *tyrR* in *E. coli* (Argaet *et al*., 1994). Accordingly, overexpression was achieved through excision of DNA upstream of *phhR*. Polymerase chain reaction (PCR) methodology was used to generate an intact *phhR* gene which was fused with the T7 translational start codon at an *Nde*I restriction site to create overexpression plasmid pJS88. *E. coli* BL21(DE3) that had been transformed with pJS88 was induced with 1 mM IPTG for 3 h to express PhhR. Whole-cell lysates obtained before and after IPTG induction were analysed by SDS–PAGE (data not shown). Overproduction of a 56 kDa protein was observed, and N-terminal amino acid sequencing confirmed its synonymy with PhhR.

Initial attempts to express *phhR* in *E. coli* during growth indicated that expression of *phhR* is highly toxic. The *Bgl*II–*Eco*RI fragment from pJS88 was cloned into the *Bam*HI–*Eco*RI site of pUC19 behind a *lac* promoter. When transformed into *E. coli* DH5α, transformants only

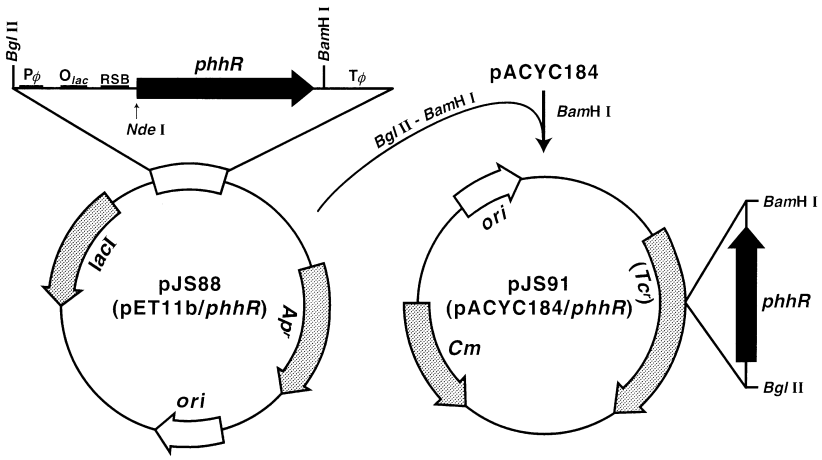


Fig. 4. Construction of PhhR overexpression plasmid pJS88 (left), and low-copy-number constitutive PhhR expression vector pJS91 (right).

achieved 'pinpoint' colony size and eventual survivor cells inevitably had lost the plasmid. Success was finally achieved by using pACYC184, a low-copy-number plasmid, to create pJS91 which carried the *Bgl*II–*Bam*HI fragment of pJS88 ligated into the *Bam*HI site of pACYC184 (Fig. 4). Analysis of 11 plasmids isolated showed that the orientation of *phhR* in each case was opposite to that of the tetracycline-resistance (Tc^R) gene. Presumably, the higher level of expression expected when driven by the Tc^R promoter still confers an intolerable level of toxicity.

Functional replacement of E. coli tyrR *with* phhR

A simple test (Camakaris and Pittard, 1973) was used to determine if *phhR* could substitute for *tyrR* as a repressor of the *aroF–tyrA* operon. *tyrR*-deficient mutants exhibit resistance to *m*-fluorotyrosine, whereas $tyrR^+$ strains exhibit sensitivity to growth-inhibitory effects of the analogue. pJS91 ($phhR^+$) was used to transform an *E. coli tyrR*-deficient background (strain SP1313). The ability of PhhR to replace TyrR was qualitatively apparent by inspection of the halo of growth inhibition on a bacterial lawn surrounding a disc containing *m*-fluorotyrosine in SP1313 ($tyrR^-$ $phhR^+$).

We also examined the ability of PhhR to replace TyrR as an activator of *mtr*, encoding a component of a tryptophan-specific transport system. The $phhR^+$ plasmid pJS91 was transformed into two *E. coli* λ lysogens (Heatwole and Somerville, 1991) which carried *mtr′–lacZ* transcriptional fusions integrated in the chromosome as single-copy fusions.

Strain SP1312 ($tyrR^+$) exhibited the expected elevation of β-galactosidase activity following growth in the presence of tyrosine, phenylalanine, or both. However, strain SP1313 ($tyrR^-$) carrying pJS91 ($phhR^+$) produced the control level of β-galactosidase activity, regardless of the presence or absence of aromatic amino acids (data not shown). Thus, PhhR appears to be incapable of replacing TyrR as an activator of *E. coli mtr*.

Autogenous regulation of phhR *in* E. coli

The *Bam*HI–*Hin*cII fragment containing the 5′ coding regions of *phhA* and *phhR* and the intervening region (see Fig. 2A) was fused to *lacZ* to give the reporter-gene construct pJS102 (*phhR′–lacZ*). This plasmid construct was introduced into the *tyrR*-negative background of strain SP1313 in the presence or absence of pACYC184 possessing a $phhR^+$ insert. The results (Table 1) demonstrated a repressive effect of $phhR^+$ upon PhhR levels as monitored by measurement of β-galactosidase activity. Since the copy number of pJS91 (the source of PhhR molecules) in this experiment is lower than the number of repressor target sites provided by the high-copy-number pJS102, and since TyrR boxes are present within seven other transcriptional units of *E. coli*, autoregulation is undoubtedly grossly underestimated because of titration of available PhhR molecules in the system.

PhhR is a positive regulator in P. aeruginosa

PhhR and TyrR form a cluster within the larger family

Table 1. Autoregulation of *P. aeruginosa phhR* in *E. coli* SP1313 ($tyrR^-$) containing pJS102(*phhR′–lacZ*).

Second plasmid[a]	Beta-galactosidase Levels[b] in Cells Grown in:		
	M9[c]	M9 + F	M9 + Y
pACYC184	550	510	589
pACYC184($phhR^+$)	362	376	372

a. pACYC184($phhR^+$) is designated pJS91 in Table 2.
b. Beta-galactosidase levels are reported in Miller Units.
c. M9 minimal medium was supplemented with 1 mM thiamine–HCl and, where indicated, 1 mM phenylalanine (F) or 1 mM tyrosine (Y).

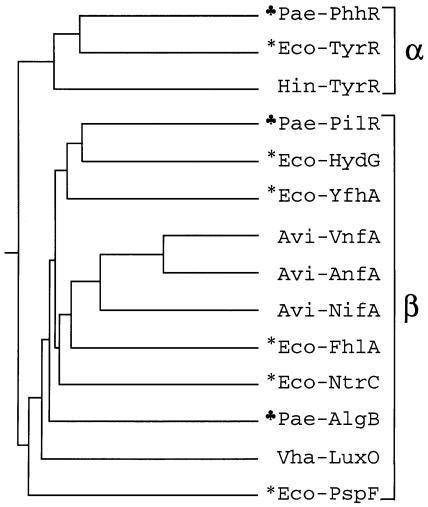


Fig. 5. Homology relationships of the central domain of *P. aeruginosa* PhhR with the central domain of other members of the σ^{54}-dependent family of transcriptional regulators. The dendrogram was generated with amino acid sequences of the central domain as defined by Morrett and Segovia (1993) by using the PILEUP program of GCG. The top three proteins form a cluster designated as subfamily α, and the remaining proteins form a larger cluster designated as subfamily β. Because of their high degree of similarity, only one of the ortholog sequences of NifA, NtrC and HydG proteins is shown. The six paralogs shown from *E. coli* and the three paralogs from *P. aeruginosa* are designated with * and ♣, respectively. *E. coli* possesses another paralog (not shown) which is closely related to *E. coli* HydG (Canellakis *et al*., 1993). It functions as ornithine decarboxylase antizyme and presumably as a transcriptional regulator of unknown genes. Abbreviations: Eco, *Escherichia coli*; Avi, *Azotobacter vinelandii*; Hin, *Haemophilus influenzae*; Pae, *Pseudomonas aeruginosa*; Vha, *Vibrio harveyi*. Functions controlled by the following regulators are given parenthetically: PhhR (phenylalanine hydroxylase), TyrR (aromatic amino acid biosynthesis and transport), VnfA (nitrogen fixation, nitrogenase-2), Anf (nitrogen fixation, nitrogenase-3), NifA (nitrogen fixation, nitrogenase-1), HydG (hydrogen oxidation), NtrC (nitrogen assimilation), PilR (synthesis of Type IV pili), AlgB (alginate production), LuxO (luminescence), FhlA (formate metabolism), YfhA (possible control of *glnB*), PspF (phage shock protein).

of σ^{54} enhancer-binding proteins, as illustrated by Fig. 5. A *rpoN* mutant of *P. aeruginosa* was assayed by Western analysis for PhhA levels of expression in order to determine whether expression of the *phh* operon is dependent upon σ^{54} like most family members, or whether it is σ^{54}-independent like *tyrR* and *luxO*. Only low basal levels of PhhA were present in the *rpoN* mutant, indicating that expression was largely σ^{54}-dependent. This, in turn, implied that *phhR* might function as an activator protein for *phhABC* transcription. *phhR* was inactivated as described in the *Experimental procedures*, and Western analysis of the effect upon PhhA levels was carried out. The results (Fig. 6) indicated that *phhR* encodes an activator, the absence of which allows only a low basal level of activity.

The small molecule, L-phenylalanine, was found to function as a physiological inducer (Fig. 7). Western analysis of PhhA showed a barely detectable band in minimal medium, compared to a prominent band when L-phenylalanine was additionally present. Carbon-source levels of L-phenylalanine were not required for induction. It is probable that L-phenylalanine is a co-activator moiety which, in combination with PhhR, forms the holo-activator moiety. It is perhaps relevant that for those transcriptional units in which TyrR functions as an activator, L-phenylalanine functions as co-activator (Pittard and Davidson, 1991).

Discussion

Anomalous repression of the phh *operon by PhhR in* E. coli

PhhR can mimic quite well the ability of TyrR to repress the *aroF–tyrA* operon at a σ^{70} promoter. This indicates that PhhR can recognize TyrR boxes and is consistent with the high similarity of the helix-turn-helix, DNA-binding domain within the carboxy-terminal segments of TyrR and PhhR. However, PhhR was unable to activate the *phh* operon in the heterologous *E. coli* background, suggesting an incompatibility between the *E. coli* RpoN and the *P. aeruginosa* σ^{54}-dependent system. The expression of PhhA from a promoter recognized by *E. coli* upstream of the native σ^{54} promoter was in fact severely depressed in constructs containing *phhR*, even in the presence of added co-activator (L-phenylalanine). In the presence of *P. aeruginosa* PhhR, an aberrant complex apparently blocks transcription initiated upstream of the σ^{54} promoter.

Emerging subfamilies within the σ^{54} enhancer-binding protein family

P. aeruginosa PhhR belongs to an outlying subgroup (which we designate subfamily α in Fig. 5) of the σ^{54} enhancer-binding protein family. All members of the family possess in common a homologous central domain, but the amino-terminal and carboxy-terminal domains may vary considerably within the family. Thus, this exemplifies a complex multi-domain protein family in which family membership is defined by a common ancestral central domain. Future subdivisions within what is termed subfamily β in Fig. 5 could probably be defined on the basis of homology

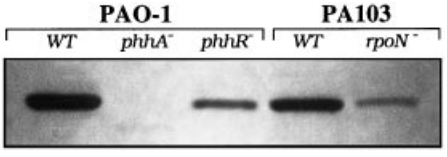


Fig. 6. Western blot analysis of *phhA* expression in mutant derivatives of *P. aeruginosa* strains PAO-1 and PA103. The proteins in crude extracts prepared from cultures grown in LB medium were separated by SDS–PAGE, and equal amounts of protein (50 μg) were applied to each lane.

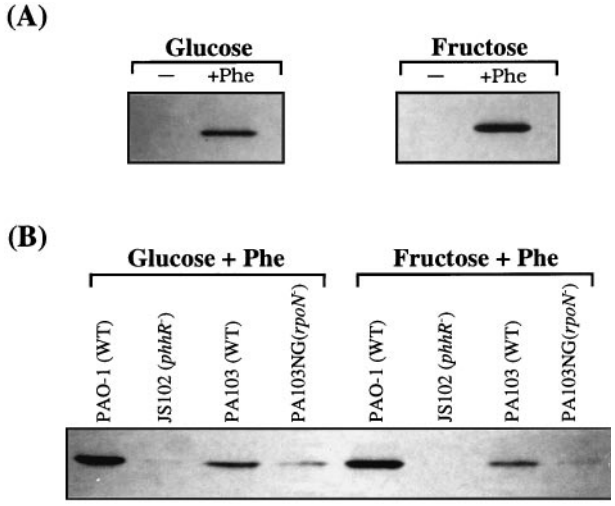


Fig. 7. Western blot analysis of *phhA* expression.
A. L-Phenylalanine as an inducer of *phhA* expression. *P. aeruginosa* PAO-1 was grown in minimal salts–glucose or minimal salts–fructose with or without addition of L-phenylalanine at a final concentration of 100 μg ml^{-1}.
B. L-Phenylalanine induction of *phhA* expression in different *P. aeruginosa* strains. Bacteria were grown in minimal–glucose or minimal–fructose media containing 100 μg ml^{-1} L-phenylalanine.

for the remaining two domains. For example, Eco–NtrC and Eco–FhlA belong to different mechanistic subgroups: the two-component regulatory system and direct response-to-small-molecules, respectively (reviewed by Shingler, 1996).

Figure 5 highlights the emerging homology relationships of selected paralog and ortholog proteins, with respect to the central domain. *E. coli* possesses at least seven paralogs, some of which diverged in a common ancestor that existed prior to speciation events that generated orthologs. Thus, the divergence of Eco–NtrC and Pae–PilR was a more recent event than the divergence of Eco–NtrC and Eco–PspF. In contrast to the ancient duplication events which generated all of the *E. coli* paralogs (or the *P. aeruginosa* paralogs) are the relatively recent duplication events generating the three paralogs that regulate three distinctly separate nitrogenase systems in *Azotobacter* (Joerger *et al*., 1989).

P. aeruginosa PhhR and *E. coli* TyrR exhibit homology in all three domains: 36% identity, amino-terminal; 52% identity, central; and 47% identity, carboxy-terminal). Curiously, the amino-terminal domain of *Haemophilus influenzae* TyrR appears to be absent. It is not known if sequencing errors might account for this, if the equivalent of the amino-terminal domain might exist separately as a different protein, or if *H. influenzae* TyrR dispenses with the functions carried out by the amino-terminal domain.

Both Eco–TyrR and Rca–NtrC exhibit deletions in the 'unique-gap region' of the central domain (Fig. 8), which correlates with their regulation of σ^{70} promoters, rather than σ^{54} promoters. This observation led to the suggestion (Morrett and Segovia, 1993) that this region of the central domain might be critical for functional interfacing with σ^{54}. Since this DNA segment of Pae–PhhR is intact with absolute retention of highly conserved residues, the foregoing hypothesis is consistent with the successful interaction of PhhR with a σ^{54} promoter. Hin TyrR, on the other hand, is likely to be deficient in interaction with σ^{54} (like *E. coli* TyrR), owing to a six-residue deletion in this region.

Intervening region of divergent transcription

Since the DNA-binding region of the carboxyl terminus of

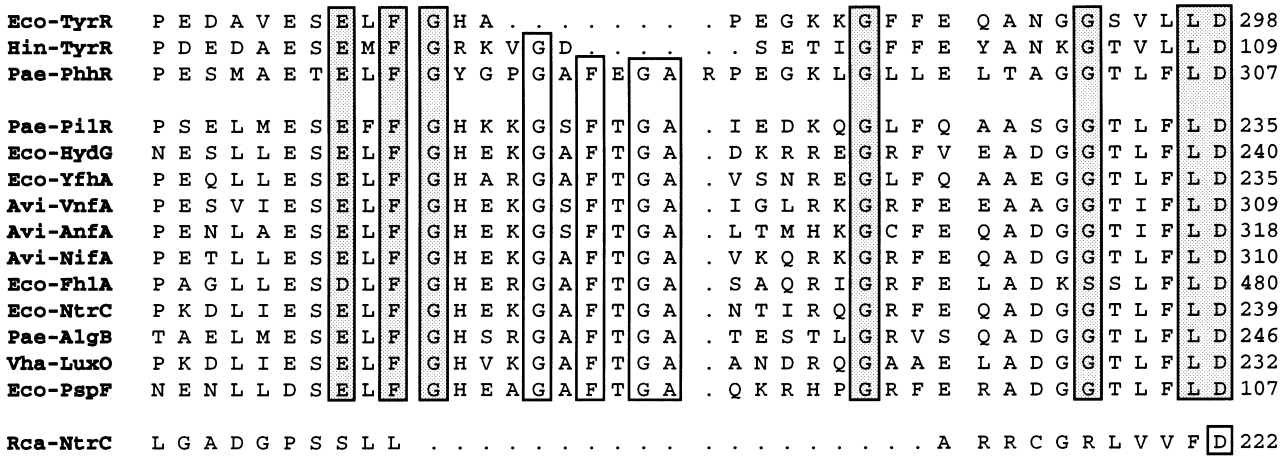


Fig. 8. Alignment of the unique-gap region in the central domains of TyrR proteins with selected homologues. Amino acid residues conserved in all of the 15 sequences that include both subfamily α (top cluster) and subfamily β (lower cluster) are in shaded boxes. Amino acid residues conserved within the gap region are shown in open boxes. Rca, *Rhodobacter capsulatus*; see the legend of Fig. 5 for other abbreviations used.

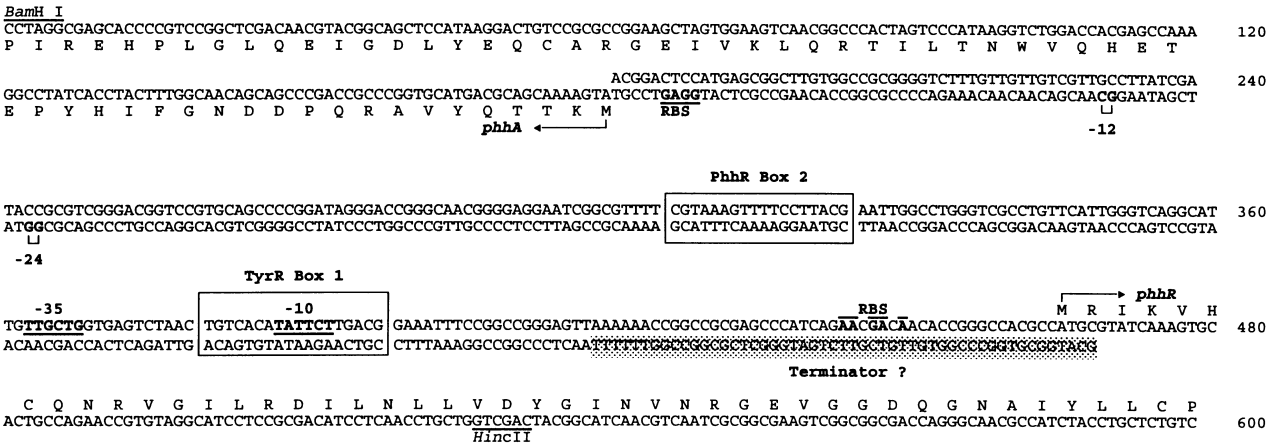


Fig. 9. The intervening sequence between the divergently transcribed *phhA* and *phhR* genes. The number at the end of each line indicates the nucleotide position. The ribosome binding sites and putative promoter sites (−12/−24 promoter for *phhA*, and −10/−35 promoter for *phhR*) are indicated. The translational start sites are indicated by arrows. Two PhhR boxes are identified. A putative rho-independent transcriptional terminator of unknown significance ($\Delta G = 67.6\ kJ\ mol^{-1}$) is shaded. Restriction endonuclease recognition sites are marked.

PhhR is identical to *E. coli* TyrR at all important residues, it is likely that PhhR binds to TyrR boxes (consensus: TGTAAAN$_6$TTTACA). This conclusion is also supported by the ability of PhhR to replace TyrR as a repressor of the *aroF–tyrA* transcriptional unit. Figure 9 shows the location of two 'PhhR boxes' which match the consensus for TyrR boxes. PhhR Box 1 overlaps the putative −10 region of the *phhR* promoter. TyrR boxes in *E. coli* occur in tandem with variable spacing (Pittard, 1996), and a TyrR hexameric molecule is thought to bind both a strong box and a weak box with DNA looping in between. PhhR Box 2 is located in the middle of the intervening region. By analogy with the TyrR system, both would be expected to behave as strong boxes if an interaction were occurring between the two boxes separated by five turns of the DNA helix. It seems probable that, by analogy with autorepression of *tyrR* in *E. coli*, both *phhR* boxes participate in the autogenous repression of *phhR* by PhhR.

In the opposite direction of transcription, the σ^{54} promoter for *phhABC* requires an upstream activator site (UAS). PhhR Box 1 may be the most likely UAS, although perhaps both boxes participate in activation of *phhA*. L-Phenylalanine, a potent inducer of phenylalanine hydroxylase, presumably is a co-activator moiety. Since an *rpoN* mutant retained a low basal level of PhhA, another promoter that is independent of σ^{54} might be present.

No motif for binding of integration host factor (IHF) (Friedman, 1988) was located in the intervening region. Therefore, this region may possess intrinsic DNA-bending capabilities.

Function of the phh *operon*

The primary function of the *phh* operon is clearly not to accommodate tyrosine biosynthesis since the feedback-inhibited cyclohexadienyl dehydrogenase that is widely distributed in Gram-negative bacteria exists for this purpose. However, the *phh* operon probably provides a fortuitous back-up capability for tyrosine biosynthesis. 'Reluctant auxotrophy' for tyrosine (Patel *et al*., 1978) can be explained as follows. Mutant deficiency of cyclohexadienyl dehydrogenase would lead to accumulation of prephenate, a potent product inhibitor of chorismate mutase. The subsequent back-up of chorismate, enhanced by a lack of early pathway control in the absence of L-tyrosine, results in passage of chorismate to the periplasm where chorismate mutase-F (W. Gu and R. A. Jensen, unpublished) and cyclohexadienyl dehydratase (Zhao *et al*., 1993) generate L-phenylalanine. Subsequent induction of phenylalanine hydroxylase completes the alternative circuit to L-tyrosine.

The established function of phenylalanine hydroxylase in mammals is for catabolism of L-phenylalanine as a carbon source. We have found that phenylalanine hydroxylase is indeed essential for use of L-phenylalanine as a sole carbon source in *P. aeruginosa*. Thus, inactivation of *phhA* resulted in an inability to use L-phenylalanine as a sole source of carbon (data not shown). However, induction of the *phh* operon under conditions in which better carbon sources (such as glucose) co-exist suggests that the *phh* operon might be dedicated to provision of some specialized compound from L-phenylalanine.

Inactivation of *phhR* resulted not only in the inability to use L-phenylalanine as a carbon source, but also in an inability to use L-tyrosine as a carbon source. Since TyrR regulates aromatic amino acid permeases in *E. coli*, we considered the possibility that the *phhR* mutant might fail to grow on L-tyrosine because of a permease deficiency. Since *m*-fluoro-tyrosine (MFT) is likely to be transported by the same system as L-tyrosine, a permease-deficient

phenotype should be resistant to growth inhibition by MFT. However, the *phhR*$^-$ mutant has an MFT-sensitive phenotype on fructose-based medium (data not shown). Therefore, PhhR might regulate steps of tyrosine catabolism. Perhaps PhhC catalyses an essential step of tyrosine catabolism?

Regulation of multiple transcriptional units by PhhR?

TyrR represses or activates eight transcriptional units in *E. coli* (Pittard, 1996). Similarly organized transcriptional units are absent or unknown in *P. aeruginosa*. However, the counterpart of the *aroF–tyrA* operon in *P. aeruginosa* would be genes encoding tyrosine-sensitive DAHP synthase and cyclohexadienyl dehydrogenase. Physiological manipulations in our laboratory have never revealed repression control of these apparently constitutive enzymes. Consistent with this, PhhR exhibits no regulatory control of either of these enzymes, judged on the basis of assessment of specific activities determined in comparisons of *phhR*$^+$ and *phhR*$^-$ backgrounds (data not shown).

Experimental procedures

Materials

The bacterial strains and plasmids used in this study are listed in Table 2. The LB and M9 formulations (Sambrook *et al*., 1989) were used as growth media for *E. coli* and *P. aeruginosa.* Pseudomonas isolation agar (Difco) was used for isolating *Pseudomonas* knockout mutants. Additions of ampicillin (100 μg ml^{-1}), chloramphenicol (40 μg ml^{-1}), kanamycin (50 μg ml^{-1}), tetracycline (25 μg ml^{-1}), mercury chloride (15 μg ml^{-1}), phenylalanine (50 μg ml^{-1}), and thiamine (17 μg ml^{-1}) were made as appropriate. Agar was added at 20 g l^{-1} for preparation of solid medium. Restriction enzymes, T4 DNA ligase, DNA-modifying enzymes (New England Biolab or Promaga) and *Taq* DNA polymerase (Perkin-Elmer) were used as recommended by the suppliers. Other biochemicals were purchased from Sigma Chemical Co. Inorganic chemicals (analytical grade) were from Fisher Scientific.

Phenylalanine hydroxylase assay

E. coli JP2255 carrying the various plasmids specified were grown in 500 ml of LB broth supplemented with ampicillin (100 μg ml^{-1}) at 37°C and harvested at the late-exponential phase of growth. The cell pellets were resuspended in 10 ml of 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol and were disrupted by sonication. The resulting extracts were centrifuged at 150 000 $\times$ *g* for 1 h at 4°C. The supernatant (crude extract) was desalted using Sephadex G-25 and used for enzyme assay. PhhA was assayed by following tyrosine formation (Nakata *et al*., 1979).

Recombinant DNA techniques

Molecular cloning and DNA manipulation, including plasmid purification, restriction enzyme digestion, ligation, and transformation, were conducted by using standard methods (Sambrook *et al*., 1989). DNA fragments were purified from agarose gel with a GeneClean kit (Bio101). Electroporation (Invitrogen) was used for simultaneous transformation of *E. coli* with two compatible plasmids.

Construction of PhhR expression vectors

For overexpression of PhhR protein, we employed the T7 expression system in *E. coli* (Novagen). We cloned the *phhR* coding region into a translational fusion vector (pET11b). PCR was used to amplify the *phhR* gene. The upper primer (5′-ATACAT**ATG**CGTATCAAAGTGCACTGC-3′) was made with a built-in *Nde*I restriction site (underlined) which allows fusion of *phhR* at the translational start site (ATG in bold). The lower primer (5′-CCTCCACCGTTTCTTTCCCAGCCT-3′) was chosen at a position 48 bases downstream of the translational stop codon. PhhR protein made from this PCR fragment was designed to be a native protein, not a fusion protein. The PCR fragment was cloned into a TA cloning vector, pCRII. The *phhR* gene was excised from pCRII with *Nde*I and *Eco*RI. The *Nde*I–*Eco*RI fragment was first ligated with an *Eco*RI–*Bam*HI adaptor to create an *Nde*I–*Bam*HI fragment which was then ligated with pET11b digested with *Nde*I and *Bam*HI to create the PhhR overexpression plasmid, pJS88 (Fig. 4).

For construction of a PhhR constitutive expression plasmid, pACYC184 was chosen as the expression vector. The pACYC184 vector has a p15A origin of replication which is compatible with most commonly used plasmids using a ColEI origin of replication, and it has a low copy number (about 20 copies/cell). A high level of PhhR produced from a high-copy-number plasmid may be toxic to the host cells. The *Bgl*II–*Bam*HI fragment carrying the *phhR* gene was excised from overexpression plasmid pJS88 and cloned into the *Bam*HI site of pACYC184, thereby interrupting the Tc^R gene (Fig. 4).

Evaluation of sensitivity/resistance to m*-fluorotyrosine (MFT)*

Three *E. coli* strains, SP1312 (*tyrR*$^+$), SP1313 (*tyrR*$^-$) carrying pJS91 (*phhR*$^+$), and SP1313 (*tyrR*$^-$) carrying pACYC184 (*phhR*$^-$), were compared for sensitivity to MFT. All three strains were first grown in M9 medium with appropriate antibiotics up to the late-exponential phase of growth and then used to swab M9 agar plates containing appropriate antibiotics. A sterile Difco concentration disk was positioned at the centre of each plate, and 10 μl of 50 μg ml^{-1} MFT was applied to the disks. The plates were then incubated at 37°C for 24 h.

Construction of phhA′–lacZ *and* phhR′–lacZ *transcriptional fusions*

To compare levels of *phhA* transcription in both pJZ9 and pJZ9-3a, plasmids pJS61Z and pJS62Z were constructed. These have a promoterless *lacZ* gene (from plasmid Z1918) fused at the *Bam*HI site within *phhA* to form *phhA′–lacZ*

Table 2. Bacterial strains and plasmids.

Strain/ Plasmid	Relevant genotype or description	Source/Reference
Strain		
E. coli		
BL21(DE3)	F$^-$ *ompT hsdS$_B$* ($r_B^- m_B^-$) *gal dcm*; with DE3, a λ prophage carrying the T_7 RNA polymerase gene	Novagen
BW545	Δ(*lacU*)*169 rpsL*	Rosentel *et al.* (1995)
DH5α	F$^-$Δ*lacU169* φ*80dlacZ*Δ*M15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 supE44*	Gibco/BRL
LE392	F$^-$ *e14*$^-$(*McrA*$^-$)*hsdR514* ($r_k^- m_k^+$) *supE44 supF58 lacY1 or* Δ(*lacIZY*)*6 galK galT22 metB1 trpR55*	Sambrook *et al.* (1989)
JP2255	*aroF363 pheA361 pheO352 tyrA382 thi-1 strR712 lacY1 xyl-15*	Baldwin and Davidson (1981)
S17-1	[RP4–2(Tc:Mu) (Km:Tn*7*) Tra(incP)] *pro hsdR recA* Tp^R Sm^R	Simon *et al.* (1983)
SP1312	*zah-735*::Tn*10*Δ(*argF–lac*)*U169*	Heatwole and Somerville (1991)
SP1312 (λSLW20)	SP1312φ(*mtr′–lacZ*$^+$)	Heatwole and Somerville (1991)
SP1313	As SP1312, but Δ(*tyrR*)	Heatwole and Somerville (1991)
SP1313 (λSLW20)	SP1313φ(*mtr′–lacZ*$^+$)	Heatwole and Somerville (1991)
P. aeruginosa		
PA103		Totten *et al.* (1990)
PA103NG		Totten *et al.* (1990)
PAO-1	Prototroph	Holloway (1955)
JS101	PAO-1(*phhA*$^-$), Hg^R	This work
JS102	PAO-1(*phhR*$^-$), Hg^R	This work
Plasmid		
pUC18	Amp^R *lac′IPOZ′*	Yanisch-Perron *et al.* (1985)
pUC19	Amp^R *lac′IPOZ′*	Yanisch-Perron *et al.* (1985)
pACYC184	P15A replicon, Cm^R Tc^R	Chang and Cohen (1978)
pET11b	T7*lac* promoter, *lacI*$^+$ Ap^R	Novagen
pRS1274	*lacZY* transcriptional fusion vector	Simons *et al.* (1987)
Z1918	Promoterless *lacZ*, Ap^R	Schweizer (1993)
pJZ9	*phhRABC*, Ap^R	Zhao *et al.* (1994)
pJZ9-3a	*phhAB*, Ap^R	Zhao *et al.* (1994)
pJS7	*phhRABC*, Ap^R	This work
pJS60	*phhABC*, Ap^R	This work
pJS61Z	*phhRA′–lacZ* transcriptional fusion, Ap^R	This work
pJS62Z	*phhA′–lacZ* transcriptional fusion, Ap^R	This work
pJS88	pET11b carrying *phhR* translational fusion at the ATG start site	This work
pJS91	pACYC184 carrying *phhR*$^+$, Cm^R	This work
pJS102	pRS1274 carrying *phhR′–lacZY* transcriptional fusion	This work
pDG106	Hg^R Km^R P15A replicon	Gambill and Summers (1985)
pJS101	*Pst*I–*Sma*I fragment of pDG106 inserted into pUC18	This work
pUFR004	ColE1 Cm^R Mob$^+$ *mob*(P)	DeFeyter *et al.* (1990)

transcriptional fusions. Plasmid pJS61Z has the same upstream sequence as plasmid pJZ9, and plasmid pJS62Z has the same upstream sequence as pJZ9-3a. Hence, the *phhA′–lacZ* fusions in pJS61Z and pJS62Z should represent the *phhA* transcriptional levels in pJZ9 and pJZ9-3a, respectively.

To study regulation of the *phhR* promoter, the *Hinc*II–*Bam*HI fragment (phhR′) was cloned into the pRS1274 *lacZY* transcriptional fusion vector at the *Bam*HI–*Sma*I site to create pJS102(*phhR′–lacZ*).

Beta-galactosidase assay

Beta-galactosidase activity was assayed under conditions of proportionality as described by Miller (1972), and specific activities are expressed in Miller units. The data are the results of at least two independent assays.

Gene inactivation

P. aeruginosa is well known for its relatively high resistance to most antibiotics, which complicates attempts to use most of the available antibiotic-resistance genes as selective markers for gene replacement. We used mercury resistance (Hg^R) as a selective marker since *P. aeruginosa* has been shown to be quite sensitive to mercury (Essar *et al*., 1990; Gambill and Summers, 1985). Our insertional inactivation technique utilizes a mobilizable suicide vector containing a truncated gene fragment (at both 5′ and 3′ ends) and an Hg^R-cassette, and this suicide plasmid is integrated into the chromosome by a single homologous recombination event. PCR was used to generate truncated fragments. To generate a ′*phhR′* (601 bp) fragment, the upper primer 5′-CCGTGTAGGCATCCT-CCGCGACAT-3′, and the lower primer 5′-CTGGAAGA-TACTGTCGAAGCCACG-3′ were used; to generate the ′*phhA′* (639 bp) fragment, we used the upper primer 5′-ACG-ACAACGGTTTCATCCACTATC-3′ and the lower primer 5′-GGACGAAATAGAGCGGTTGCAGGA-3′. The PCR-generated fragments were cloned into pCRII (a TA cloning vector) and then excised with *Eco*RI. The *Eco*RI fragments were subsequently cloned into the *Eco*RI site of pUFR004 (a mobilizable suicide vector) to create pUFR/′*phhA′*, and

the Hg^R *Hin*dIII-cassette from pJS101 was inserted into the *Hin*dIII site of pUFR/′*phhA*′ to create pUFR/′*phhA*′/Hg^R. pUFR/′*phhR*′/Hg^R was created in a similar fashion. These plasmids were then used to transform *E. coli* S17-1 (a mobilizing strain). S17-1 harbouring either pUFR/′*phhA*′/ Hg^R or pUFR/′*phhR*′/Hg^R was used as the donor in biparental mating with *P. aeruginosa* performed as described by Simon *et al.* (1983). Donor and recipient cells were grown in LB broth to an OD_{600} of about 1.0 (*E. coli* S17-1 at 37°C and *P. aeruginosa* PAO-1 at 42°C), mixed (0.5 ml volume of each) in a 1.5 ml microcentrifuge tube, and pelleted by centrifugation. The mating mixture was carefully resuspended into 0.2 ml of LB broth and spread onto a sterile nitrocellulose filter (0.45 μm pore size) resting on a prewarmed LB agar plate. The plates were incubated for 16–24 h at 37°C, and then cells were removed from the filter by an inoculation loop and resuspended by vortexing into 0.5 ml of LB broth. Aliquots (10, 20, 50, and 100 μl) of the cell suspension were spread onto Pseudomonas isolation agar plates containing 15 μg of $HgCl_2$. The plates were incubated overnight and Hg^R colonies were isolated. Interruption of the targeted gene by integration of the suicide vector was confirmed by Southern blot analyses of Hg^R isolates.

SDS–PAGE and Western blot analysis

SDS–PAGE (12%) was performed with the Mini-PROTEAN II Cell (Bio-Rad) according to the method of Laemmli (1970). 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 5–10 μl were loaded onto two SDS–acrylamide gels. After separation of the proteins by electrophoresis, one gel was stained with Coomassie brilliant blue and the other gel was used for blotting. When crude extracts were used, equivalent amounts of protein were loaded in each lane. Western blots were performed according to Towbin *et al.* (1979). The proteins were eletrophoretically transferred onto nitrocellulose membranes and reacted with the polyclonal antibodies raised against PhhA in a rabbit.

N-terminal amino acid sequencing

PhhR protein overproduced in *E. coli* BL21(DE3)/pJS88 following induction by 1 mM IPTG for 3 h was first separated from the whole lysate by SDS–PAGE. The proteins were then blotted to a PVDF membrane (Bio-Rad) and were stained with Coomassie brilliant blue (Sigma). The band corresponding to PhhR was excised from the membrane and used for sequencing with an Applied Biosystems Model 407A protein sequencer with an on-line 120A phenylthiohydantoin analyser in the Protein Core Facility at the University of Florida.

DNA sequencing and data analysis

Sequencing of the *phhR* region was performed by the DNA Sequencing Core Laboratory of the University of Florida. The nucleotide sequence and the deduced amino acid sequence were analysed by using the updated version of sequence analysis software package offered by the Genetics Computer Group (GCG) of the University of Wisconsin (Devereux *et al.*, 1984).

Nucleotide sequence accession number

The nucleotide sequence reported in this paper has been assigned GenBank Accession No. U62581.

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