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aph(3′)-IIb, a Gene Encoding an Aminoglycoside-Modifying Enzyme, Is under the Positive Control of Surrogate Regulator HpaA

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Pseudomonas aeruginosa **harbors a chromosomal aminoglycoside phosphotransferase gene, *aph(3′)-IIb*, which confers *P. aeruginosa* resistance to several important aminoglycoside antibiotics, including kanamycin A and B, neomycin B and C, butirosin, and seldomycin F5. The *aph(3′)-IIb* gene has been found to be regulated by an AraC-type transcriptional regulator (HpaA) encoded by a gene located upstream of the *aph(3′)-IIb* gene. In the presence of 4-hydroxyphenylacetic acid (4-HPA), HpaA activates the expression of *aph(3′)-IIb* as well as that of the *hpa* regulon which encodes metabolic enzymes for the utilization of 4-HPA. *hpaA* and *aph(3′)-IIb* form an operon, and in response to the presence of 4-HPA, the wild-type *P. aeruginosa* strain PAK (but not its *hpaA* mutant strain) displays increased resistance to neomycin. A survey of 39 clinical and 19 environmental isolates of *P. aeruginosa* demonstrated in all of them the presence of an *hpaA-aph* gene cluster, while 56 out of the 58 isolates are able to utilize the 4-HPA as a sole carbon source, suggesting a feature common to *P. aeruginosa* strains. Interestingly, a larger portion of clinical isolates than environmental isolates showed 4-HPA-induced resistance to neomycin. The *aph(3′)-IIb* gene product is likely to function as a metabolic enzyme which has a cross-reactivity with aminoglycosides. These findings provide new insight into the possible mechanism of *P. aeruginosa* antibiotic resistance.**

Pseudomonas aeruginosa, a gram-negative bacterium, is an opportunistic human pathogen. It is the major causative pathogen for morbidity and mortality in cystic fibrosis (CF) and burn patients as well as in immunocompromised patients (1, 8). This pathogen can survive in most environmental niches and infects a variety of hosts, demonstrating a tremendous capacity for adaptation and complex regulatory machinery (11, 12, 14).

P. aeruginosa is able to utilize 4-hydroxyphenylacetic acid (4-HPA) and 3,4-dihydroxyphenylacetic acid (3,4-DHPA) (5, 6) and catabolize some of the aromatic biogenic amines (such as tyramine and dopamine) found in mammalian nervous systems. The genetics of this particular metabolic pathway for *Escherichia coli* have been well described previously (3, 4). *E. coli* strains B, C, and W are able to utilize 3-hydroxyphenylacetic acid (3-HPA), 4-HPA, and 3,4-DHPA as alternative carbon sources through an *hpa* pathway consisting of the hydroxylation of 3-HPA or 4-HPA and the subsequent *meta* cleavage of 3,4-DHPA, which are encoded by the *hydroxylase* and *meta* operons, respectively (4). The *hydroxylase* operon is positively regulated by HpaA, an AraC family regulator, while the *meta* operon is repressed by HpaR, a negative regulator. Both HpaA and HpaR respond to the *hpa* substrate molecules (including 3-HPA and 4-HPA) to activate *hpa* regulon expression (19, 20). *P. aeruginosa* harbors homologues of the *E. coli hpa* pathway genes (29); however, there is no report on the function and regulation of these genes.

P. aeruginosa also harbors an array of aminoglycoside-modifying genes, enabling enzymatic inactivation of aminoglycosides by acetylation (7, 24), adenylation (25), or phosphorylation (APH) (9). These genes are either plasmid borne or chromosomally localized; in the latter case, a transposon-mediated mechanism has been suggested to be responsible for spreading the genes into this species (7, 18, 24). There has been no report of a study suggesting a possible correlation (either genetic and physiological) between an aminoglycoside-modifying gene and the HPA metabolism pathway.

In this report, *aph(3′)-IIb*, an aminoglycoside-phosphotransferase gene of *P. aeruginosa* (9), is shown to form an operon structure with its upstream *hpaA* homologue. The operon is activated by the HpaA homologue in response to the presence of 4-HPA, enabling *P. aeruginosa* to utilize 4-HPA as a sole carbon source. Activation of the *hpa* regulon in response to its substrate also leads to increased *aph(3′)-IIb* expression, resulting in elevated resistance to aminoglycoside antibiotics. These results may help partially explain the intrinsic as well as adaptive aminoglycoside resistance in *P. aeruginosa*.

MATERIALS AND METHODS

Materials, strains and media. All strains and plasmids used in this study are listed in Table 1. *E. coli* and *P. aeruginosa* strains were grown at 37°C in Luria-Bertani (LB) broth or M63 minimal medium (21). The M63 medium salt was supplemented with 1 μg of vitamin B_{12}/ml and 0.2% of glycerol unless specified otherwise. The 4-hydroxylphenylacetate (4-HPA) and 3-hydroxylphenylacetate (3-HPA) were purchased from Sigma (St. Louis, Mo.). They were dissolved in water and adjusted to pH 7.0 with KOH before being added to the culture medium. Antibiotics and concentrations were as follows: for *E. coli*, ampicillin (100 μg/ml), kanamycin (50 μg/ml), tetracycline (20 μg/ml), gentamicin (10 μg/ml), spectinomycin (50 μg/ml), and streptomycin (25 μg/ml); and for *P. aeruginosa*, tetracycline (100 μg/ml), gentamicin (200 μg/ml), carbenicillin (150 μg/ml), spectinomycin (200 μg/ml), and streptomycin (200 μg/ml).

Mutations and plasmid construction. Mutant strains of *hpaA* and *aph* in a strain PAK background were constructed using a sucrose selection suicide delivery system as described previously (10). For *hpaA* mutation, specific primers HpaA5 and Aph3 (Table 1) were used to amplify a 1.5-kb *hpaA*-containing region. The PCR product was ligated into TA cloning vector pCR2.1-TOPO (Invitrogen) to give rise to pWC003, from which the *hpaA*-containing fragment

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TABLE 1. Strains, plasmids, and primers

Strain, plasmid, or primer	Genotype or description	Source or reference
Strains		
E. coli		
DH5α	F$^-$ ϕ80 δ*lacZ* ΔM15 *endA1 recA1 hsdR17 supE44 thi-1 relA1* Δ(*lacZYA-argF*) *gyrA96 deoR*	GIBCO-BRL
P. aeruginosa		
PAK	Wild-type clinical isolate	David Bradley
PAK1-3	A spontaneous mutant derivative of PAK, Neo^r	31
PAK(*aph*)	PAK with *aph* mutated by gentamicin cassette insertion	This study
PAK(*hpaA*)	PAK with *hpaA* mutated by Ω insertion	This study
Plasmids		
pUCP19	Broad-host-range plasmid, Cb^r	32
pWC001	2-kb DNA containing *aph* insert cloned into pUCP19	This study
pEx18Tc	Sucrose selection suicide delivery plasmid, Tc^r	10
pExaph	pEx18Tc containing 1.7-kb *Bam*HI fragment from pWC001	This study
pExaphG	pExaph with gentamicin cassette inserted at *Nsi*I site disrupting *aph* gene	This study
pDN19lacΩ	A *lacZ* fusion plasmid vector, IncP, Sp^r/Sm^r/Tc^r	30
pCR2.1-TOPO	TA-cloning vector, Ap^r Km^r	Invitrogen
pWC003	1.5-kb PCR product (HpaA5-Aph3) of *hpaA* cloned into pCR2.1-TOPO	This study
pWC011	pDN19lacΩ containing 1.6-kb fragment from pWC003, *aph*::*lacZ* fusion	This study
pWC012	pDN19lacΩ containing 1.1-kb fragment from pWC003, *hpaA*::*lacZ* fusion	This study
pWC013	pDN19lacΩ containing 1.6-kb fragment from pWC003, PA4121::*lacZ* fusion with intact *hpaA*	This study
pWW001	1.0-kb PCR product (Aph5-Aph3) of *aph* cloned into pCR2.1-TOPO	This study
pWC014	pDN19lac14 containing 1.1-kb fragment from pWW001, *aph*::*lacZ* fusion	This study
pWC018	PA4121::*lacZ* fusion, without *hpaA*	This study
pEx18Ap	Sucrose selection suicide delivery plasmid, Ap^r	10
pExhpaA	pEx18Ap containing *Xba*I/*Sac*I fragment from pWC003	This study
pWC021	pExhpaA with its *Bgl*II fragment replaced by Ω insertion	This study
Primers		
HpaA5	5′ CGTTGACGATCACGTAGCCGGCGACAT 3′	
Aph5	5′ GAGCGCCAGCCGATCCCCAACATCAAC 3′	
Aph3	5′ CTCGCCAGCGGTAGCCGGCAAGGTAGT 3′	

was subcloned into an *Xba*I/*Sac*I site of *sacB*-containing vector pEx18Ap, resulting in pExhpaA. Then an Ω cassette was used to replace the *Bgl*II fragment of the *hpaA* gene in pExhpaA, and the resulting plasmid (pWC021) was used to transform wild-type PAK. Spectinomycin-streptomycin-carbenicillin-resistant single-crossover colonies were selected on plates followed by plating on LB agar containing spectinomycin-streptomycin and 5% sucrose. The resulting double-cross mutants were confirmed by PCR using primers Aph5 and Aph3.

The *aph* mutant was generated in a similar fashion. pWC001, a clone containing a partial *hpaA-aph* region, was digested with *Bam*HI, and the resulting fragment was inserted into the same site of sucrose selection plasmid pEx18Tc to generate pExaph. pExaph was then digested with *Nsi*I, and a gentamicin cassette was inserted. The resulting plasmid, pExaphG, was used to generate an *aph* knockout mutation by sucrose selection as described above. The *aph* mutation was confirmed by Southern blot analysis.

A 1.6-kb *Eco*RI fragment (containing an intact *hpaA* gene in the middle as well as the N terminus of *aph* and the open reading frame [ORF] PA4121 in the opposing direction at the 5′ and 3′ ends, respectively) was isolated from pWC003 and inserted into *lacZ* fusion vector pDN19lacΩ. The resulting plasmids pWC011 and pWC013 encode APH-LacZ and PA4121-LacZ fusions, respectively. To construct the *aph-lacZ* fusion without an *hpaA* gene, a 1.0-kb fragment was amplified using primer set Aph5-Aph3 and cloned into pCR2.1-TOPO to generate pWW001. A 1.1-kb *Eco*RI fragment was isolated from pWW001 and inserted into pDN19lacΩ, generating an *aph*::*lacZ* fusion construct named pWC014. To construct the PA4121-LacZ fusion without an *hpaA* gene, a *Bam*HI-*Bgl*II fragment from pWC003 was inserted in front of the promoterless *lacZ* gene in pDN19lacΩ, generating pWC018. Similarly, a 1.1-kb *Eco*RI-*Bgl*II fragment from pWC003 was used to construct *hpaA*::*lacZ* fusion plasmid pWC012.

Neomycin resistance tests. Two methods were used to determine inducible resistance to neomycin. First, a double-disk diffusion test was used for qualitative assays. Specified amounts of antibiotics and HPA (3-HPA or 4-HPA) solutions were dropped onto round sterile filter paper disks (7 mm in diameter) and air dried. Fresh bacterial cultures were spread onto M63 agar plates with sterile cotton swabs, and HPA disks were placed on the plates. Empty disks were used as controls. After 5 h of incubation at 37°C, the antibiotic disks were placed ca. 1.25 cm away from the HPA disks. Plates were further incubated at 37°C, and the inhibition zones were observed after 15 to 20 h. For a quantitative assay, bacteria were grown in LB broth overnight and the cell density was determined by measuring the optical density at 600 nm. After serial dilutions, bacterial cells were inoculated into the antibiotic gradients at a final density of 5×10^5 cells/ml. MICs were determined after 36 h of incubation at 37°C without agitation. All MIC tests in this report were done by using M63 medium containing 0.2% glycerol supplemented with various concentrations of 4-HPA.

Miscellaneous assays. *P. aeruginosa* strains harboring various *lacZ* fusion constructs were grown overnight in M63 medium with or without 5 mM HPA unless specified otherwise, and their β-galactosidase activities were measured as described previously (16). Growth curves of the PAK strain and various mutant derivatives were generated by measuring the optical density at 600 nm of the cultures in M63 medium supplemented with 10 mM 4-HPA as a sole carbon source.

RESULTS

Isolation of *aph* and its upstream putative regulatory gene *hpaA*. Wang et al. have previously described PAK1-3, a multidrug-resistant *P. aeruginosa* strain derived from strain PAK (31). As part of an effort to identify the mutant gene(s) responsible for the antibiotic resistance phenotype, we constructed a PAK1-3 genomic clone bank and introduced it into the PAK strain to select for clones conferring neomycin resistance (at 200 μg/ml). A total of seven positive colonies were

A.

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PA        MPERQPIPNINIGQVYDQRYADAEVHYDALGNLAGFFGRNMPAHRHDRFFQVHYVKNGAV
          M +RQ I NI+I + YD+      +VHY +    +A F     +P HRH+++FQ+H++ +G +
E.coli W  MCDRQ-IANIDISKEYDESLGTDDVHYQSFARMAPFLASMLP-HRHEQYFQMHFLNSGQI

PA        RVYLDERQYLESGPMFFLTPPTVPHAFVTEADADGHVLTVRQQLVWSLTEAEPGLAPGPQ
           + LD+ +Y     P+F LTPP+VPHAF+TE+DADGHVLTVR+ L+W L E     L PG +
E.coli W  ELQLDDHRYSVEAPLFVLTPPSVPHAFITESDADGHVLTVREDLIWPLLEV---LYPGTR

PA        VAPACVALGNLEGGAAEEAARLALLFEQLRSESMASRSGRQQALLALTRLVMISLLR-LS
                 +          E A L       +  ES+     GR+   L   L + V    LLR
E.coli W  ETFGLPGICLSLADKPDELAALEHYWQLIERESVEQLPGREHTLTLLAQAVFTLLLRNAK

PA        ANSVAARPMRHEDLQIFQRFNALIEERYAEHWPLSLYASRIGVTEARLNDVCRRIADLPS
           +  AA  MR E L++FQRFN LIE  + +HW +  YA+ + +TE+RL D+CRR A+ P
E.coli W  LDDHAASGMRGE-LKLFQRFNMLIESHFHQHWTVPDYANELHITESRLTDICRRFANRPP

PA        KRLVYERLMQESKRLLLFTGGSVNEICYQLGFKDPAYFSRFFVRYAGLTPSAYRQRQ
          KRL+++R ++E+KRLLLF+ +VN I +QLGFKDPAYF+RFF R G +PSAYR ++
E.coli W  KRLIFDRQLREAKRLLLFSDNAVNNIAWQLGFKDPAYFARFFNRLVGCSPSAYRAKK
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B.

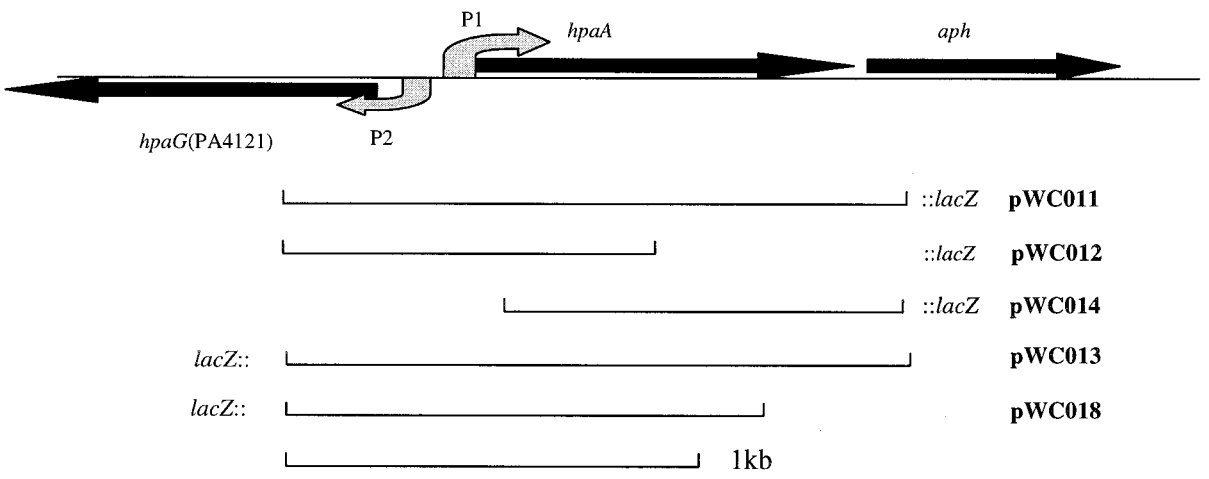

FIG. 1. (A) Alignment of HpaA from *P. aeruginosa* and *E. coli*. Letters between two lines of a sequence designate identical amino acids; a plus sign indicates two similar amino acids. (B) A schematic representation of the *hpaA-aph* locus in *P. aeruginosa* and the structures of *lacZ* fusion plasmids pWC011, pWC012, pWC013, pWC014, and pWC018. Filled straight arrows represent ORFs and transcription direction; curved arrows shaded gray show the putative promoters.

obtained, from which the plasmids were isolated and analyzed by restriction enzyme digestion and sequencing. A single gene, *aph(3′)-IIb* (referred to in shortened form as *aph* hereafter), was found to be responsible for the resistance in all the clones. The *aph* gene encodes a 268-amino-acid-long aminoglycoside-phosphotransferase with 51.7% identity to APH(3′)-IIa from Tn*5* (9). In all seven positive clones that we had isolated, the *aph* gene was preceded by a putative regulatory gene which is a homologue of *hpaA* from *E. coli* W (and which we call *hpaA* as well in this report).

HpaA belongs to an AraC regulatory protein family and is required to activate the metabolic pathway genes of alternative carbon source 4-HPA in certain *E. coli* strains (20). As shown in Fig. 1A, HpaA proteins from *E. coli* and *P. aeruginosa* have a significant amount (63%) of sequence similarity. A series of HPA utilization genes were found around the *hpaA* locus (homologous to their counterparts in *E. coli* W) (19). Also in similarity to findings for *E. coli*, these genes form two separate operon-like structures, namely, the *hydroxylase* and *meta* operons. *E. coli* W harbors a positive regulator (HpaA) and a repressor (HpaR), controlling the *hydroxylase* operon and the *meta* operon, respectively. In *P. aeruginosa*, however, there is no HpaR homologue; instead, two AraC-type regulator genes were found in the *hpa* operons, namely, *hpaA* and PA4094 (Fig. 2). On the basis of the facts that PA4094 is adjacent to the *hydroxylase* operon (*hpaBC*), that *hpaA* is upstream of the *meta* operon, and that these two operons are far (ca. 35 kb) apart (Fig. 2), it is reasonable to speculate that in *P. aeruginosa*, PA4094 and *hpaA* regulate the two respective operons. The *aph* gene, seemingly so irrelevant to this pathway, is located

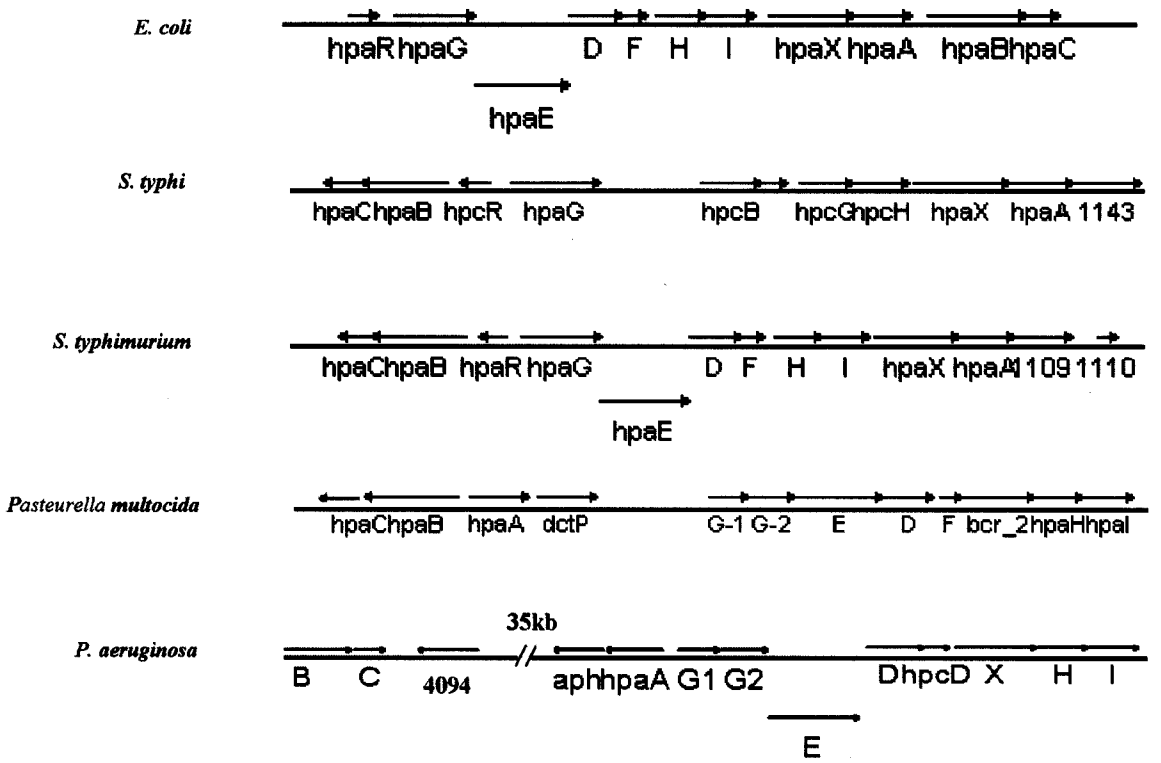

FIG. 2. Genomic structures of *hpa* regulons found in various microorganisms. All the ORFs were drawn according to their actual sizes within each regulon. Letters B, C, D, E, F, H, I, and X stand for ORFs *hpaB*, *-C*, *-D*, *-E*, *-F*, *-H*, *-I*, and *-X*, respectively.

immediately downstream of *hpaA*. As shown in Fig. 1B, the close proximity of *hpaA* and *aph* (51 bp apart), and the absence of any promoter-like element immediate upstream of the *aph* gene, introduces the possibility that these two genes might form an operon structure.

Regulation of the *aph* gene by the autoregulator HpaA. We first tested the expression of *hpaA* by using an *hpaA*::*lacZ* fusion plasmid (pWC012). Strain PAK and the *hpaA* mutant containing pWC012 were grown in M63 minimal medium supplemented with 5 mM 4-HPA or left untreated, and β-galactosidase assays were performed. As shown in Fig. 3, no differences in the β-galactosidase activities were observed in the PAK and the *hpaA* backgrounds when cells were grown without 4-HPA. In the presence of 5 mM 4-HPA, however, a significant induction of *hpaA* expression was observed in wild-type PAK and no induction was detected in the *hpaA* background, suggesting that HpaA activates its own expression in response to the presence of the 4-HPA substrate.

Next, we tested the effect of *hpaA* on *aph* expression. As shown in Fig. 3, when induced by 5 mM 4-HPA the expression of *aph*::*lacZ* in the presence of *hpaA* (pWC011) (as measured by β-galactosidase activity levels) increased more than twofold. In the absence of *hpaA* (i.e., pWC014 in an *hpaA* mutant background), however, the β-galactosidase activity did not respond to the induction by 4-HPA and remained at a level very close to that of pWC011 without 4-HPA induction, which was significantly higher than that of the vector control. These results suggested that there are two layers of control over *aph* expression: one provided by the *hpaA* promoter, which responds to the presence of 4-HPA, and another one probably residing in the *hpaA-aph* intergenic region and acting constitutively. In agreement with this hypothesis, the expression of *aph*::*lacZ* (pWC014) in an *hpaA* mutant background did not decrease in the presence or absence of 4-HPA, suggesting that HpaA exerts no control on the second *aph* promoter.

4-HPA induces neomycin resistance in *P. aeruginosa*. Since *aph* transcription can be activated by adding 5 mM 4-HPA in the culture medium, we further tested whether the presence of 4-HPA in culture medium changes the level of bacterial resistance to neomycin. This was first investigated with a double-disk diffusion assay. As shown in Fig. 4, the presence of 4-HPA decreased the size of the inhibition zones formed by neomycin on the PAK bacterial lawn in a concentration-dependent fashion. However, 4-HPA failed to interfere with the formation of a neomycin inhibition zone on either *hpaA* or *aph* mutant strains. Apparently 4-HPA is able to induce *P. aeruginosa* aminoglycoside resistance in an HpaA-dependent manner.

The ability of 4-HPA to induce *aph* expression (and, hence, bacterial resistance to neomycin) was further characterized by a MIC test. A neomycin gradient was generated in M63 minimal medium supplemented with 4-HPA at concentrations ranging from 0 to 10 mM. Overnight cultures of PAK, *hpaA*, and *aph* strains were inoculated into neomycin gradients at final densities of 5×10^5 cells/ml, and bacterial growth was scored after 24 and 36 h of incubation. As shown in Table 2 for the wild-type strain PAK, increasing the concentration of 4-HPA from 0 to 10 mM led to increases (from 30 to 250 μg/ml) in the level of bacterial resistance to neomycin. Under

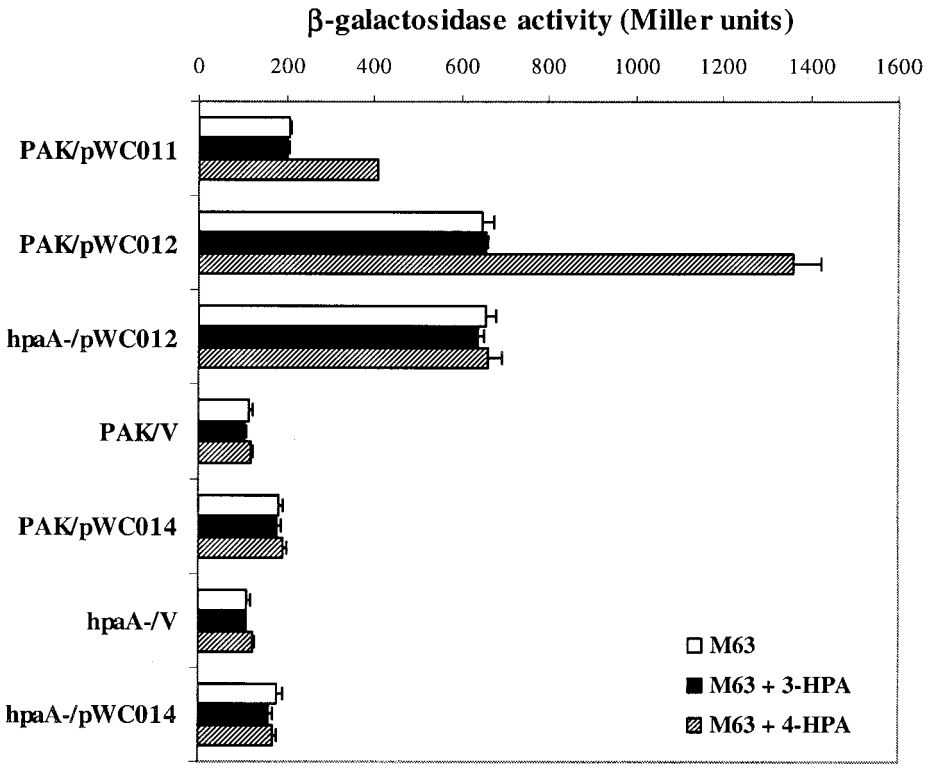

FIG. 3. Induction of *hpaA* and *aph* in *P. aeruginosa* with 4-HPA requires the presence of intact *hpaA* genes. Cells were grown overnight in M63 medium with or without supplementation of 5 mM 3-HPA or 4-HPA. Data represent the averages of the results of six independent β-galactosidase activity experiments. pWC011, pWC012, and pWC014 are *lacZ* fusion plasmids for *hpaA-aph*, *hpaA*, and *aph*, respectively (Fig. 1B); PAK/V and hpaA-/V are vector controls containing pDN19lacΩ.

the same conditions, no induction of resistance was observed in either an *hpaA* or *aph* mutant background. The neomycin resistance level for the *aph* mutant strain was (as expected) dramatically decreased, dropping from 30 μg of neomycin/ml for the wild-type strain to 5 μg/ml for the *aph* mutant strain, suggesting that the *aph* gene does play an important role in the intrinsic resistance of *P. aeruginosa* to aminoglycosides. For the *hpaA* mutant strain, an apparent defect in bacterial growth in 4-HPA-containing minimal medium was observed. After an extended period (36 h) of incubation, which enabled us to score the growth, no induction of neomycin resistance by 4-HPA was observed. However, this resistance level (30 μg of neomycin/ml) was still significantly higher than that for the *aph* mutant strain (5 μg of neomycin/ml), supporting the previous conclusion that low-level constitutive expression of *aph* does exist.

HpaA controls metabolism of 4-HPA, while APH is not required. We were intrigued by the fact that *aph* is a total stranger to the *hpa* regulon, since none of the *hpa* regulon-harboring bacteria contained the *aph* homologue. To determine whether HpaA regulates the *meta* operon, we analyzed the requirement of this special gene cluster in the metabolism of 4-HPA as a sole carbon source.

Strains of PAK, *aph*, and *hpaA* individually or the *hpaA* mutant harboring pWC011 or pWC014 were inoculated into M63 minimal medium with or without 10 mM 4-HPA as the sole carbon source. The results shown in Fig. 5 encouraged several conclusions. (i) The *P. aeruginosa* PAK strain was capable of utilizing 4-HPA as a sole carbon source for growth. (ii) The PAK strain with an *hpaA* mutation lost the ability to utilize 4-HPA, and this defect was complemented by pWC011 carrying an intact copy of *hpaA* but not by pWC014 lacking the *hpaA* gene. (iii) An *aph* mutation had no effect on the bacterial ability to utilize 4-HPA as the sole carbon source. While comparing the growth levels of the PAK strain in M63 medium with glucose, glycerol, or 4-HPA as a sole carbon source at the same molar concentrations, we found that 4-HPA served as the best carbon source, giving the PAK strain the fastest growth rate and highest stationary phase cell density (data not shown).

To test the role of HpaA in *meta* operon regulation, we constructed two PA4121::*lacZ* fusion plasmids, pWC013 and pWC018, for which PA4121 is the first gene of the *meta* operon in *P. aeruginosa* and shares homology with *E. coli* HpaG. pWC013 contains an intact copy of *hpaA*, while pWC018 does not have the *hpaA* gene (Fig. 1B). PAK and *hpaA* mutant strains containing these two plasmids were grown in M63 medium supplemented with 5 mM 4-HPA or left untreated, and β-galactosidase activities were measured. In similarity to the results seen with *hpaA* and *aph*, expression of PA4121 is activated by the HpaA in a 4-HPA-dependent manner (Fig. 6). A mutation in the *hpaA* gene abolished this induction, and this defect was complemented by the *hpaA* gene in pWC013. These results indicate that the *meta* operon of *P. aeruginosa* is posi-

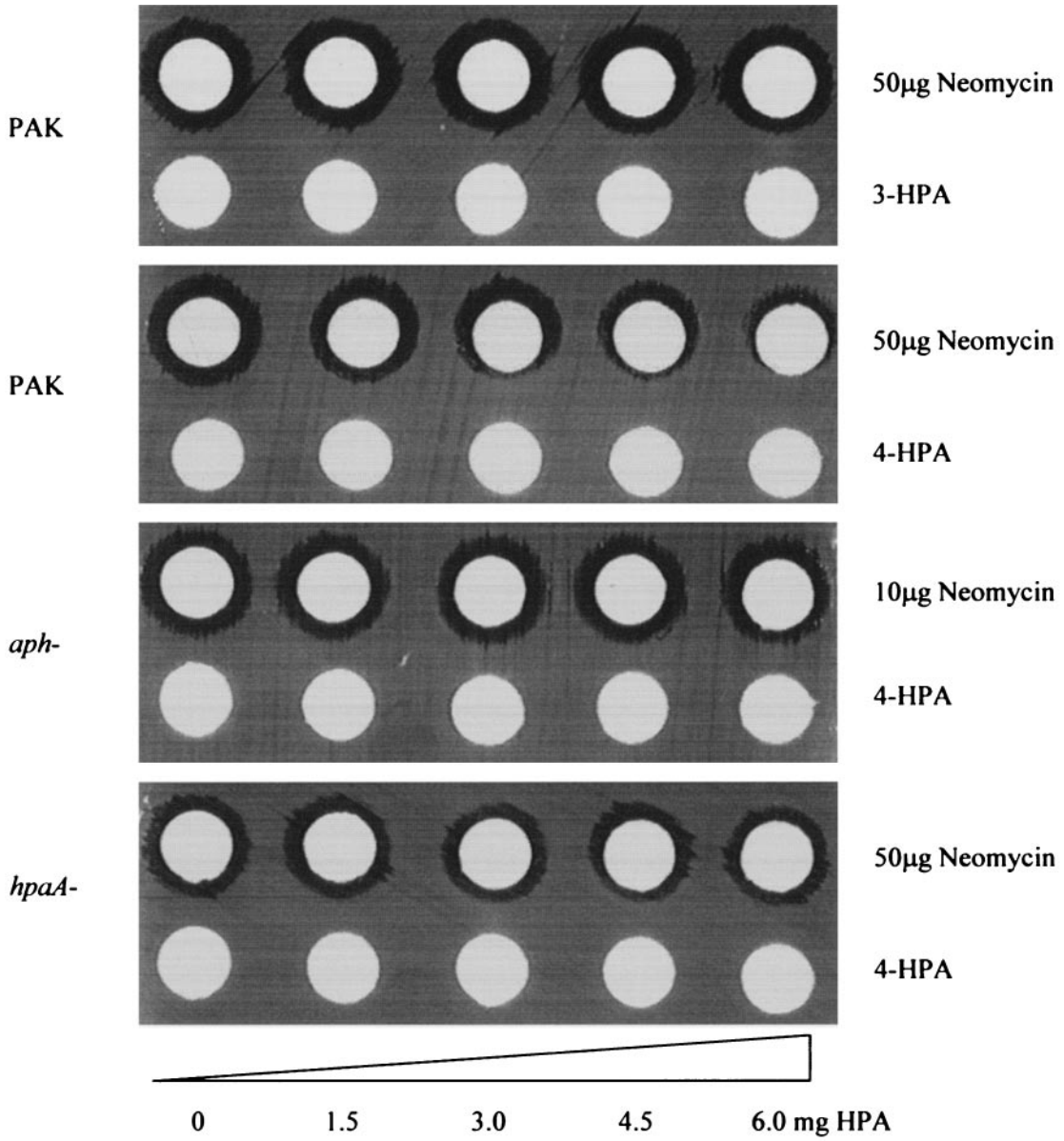

FIG. 4. The results of a double-disk diffusion assay show that neomycin resistance in *P. aeruginosa* can be induced by the presence of 4-HPA in a concentration-dependent manner. This result was not observed for the isogenic *aph* or *hpaA* mutant strains or when 3-HPA is used as inducer. The amounts of neomycin and 3- or 4-HPA used in these tests are indicated (see Materials and Methods for details).

tively regulated by HpaA (unlike the results seen with *E. coli* W, in which it was repressed by HpaR).

An unmarked deletion mutant of *hpaA* in the strain PAK background behaved exactly the same as the insertional mutant with respect to its basal and inducible (by HPA analogues) levels of resistance to neomycin as well as with respect to the expression of *hpaA* downstream genes (namely, *aph* and PA4121) (data not shown). Therefore, genes carried on the Ω fragment did not contribute to the neomycin resistance.

TABLE 2. MICs of neomycin for PAK and mutants

Strain	MIC (μg/ml) of neomycin supplemented with:		
	No 4-HPA	1 mM 4-HPA	10 mM 4-HPA
PAK	30	130	250
PAK(*aph*)	5	5	5
PAK(*hpaA*)	30	30	30

The 4-HPA analogue 3-HPA is incapable of inducing the *hpaA-aph* operon. The results of studies with *Pseudomonas putida* and *Acinetobacter* spp. have suggested that fluorescent pseudomonads utilize 3-HPA and 4-HPA by two different pathways and that these two analogous compounds share the same pathway in *E. coli* (4, 28). We first tested the growth of PAK and another standard *P. aeruginosa* strain (PAO1) on M63 medium with 3-HPA as the sole carbon source. Surprisingly, after 36 h of incubation at 37°C, no apparent growth was

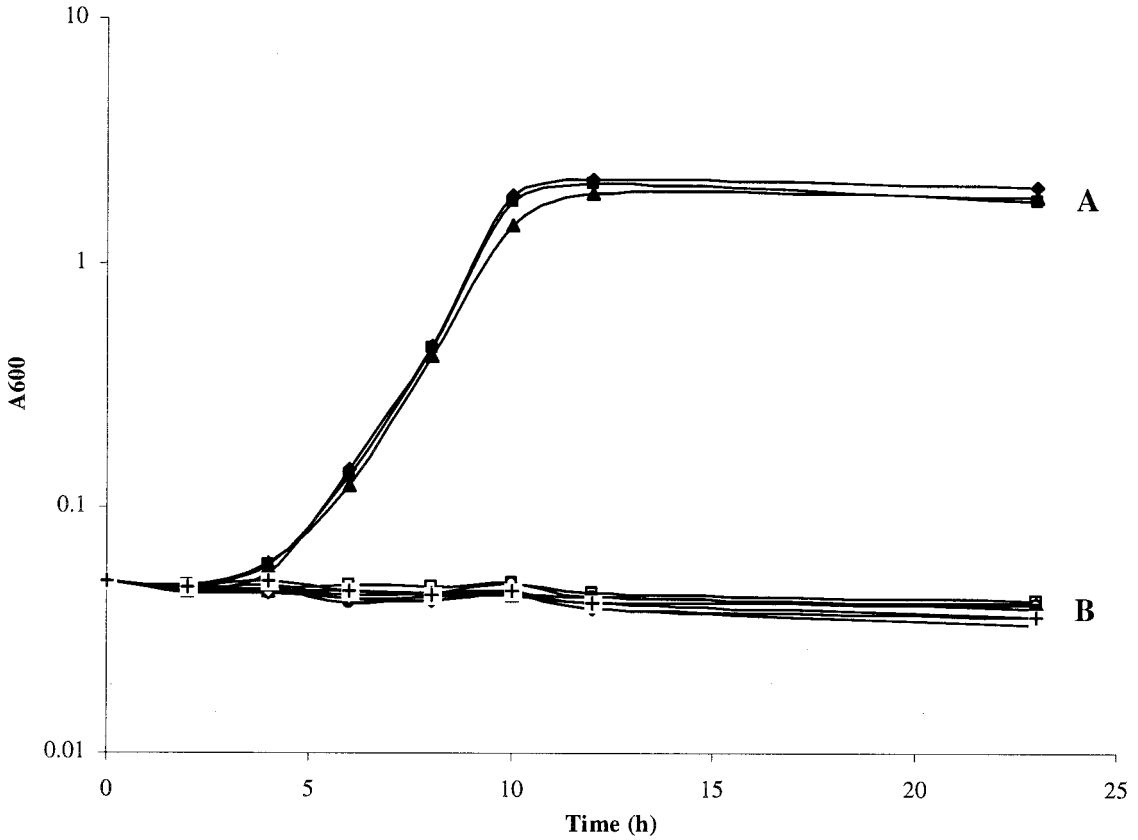

FIG. 5. Growth curves of strains PAK, PAK(*aph*), and PAK(*hpaA*) as well as those of strain PAK(*hpaA*) containing pWC011 (*hpaA* clone) or pWC014 (control) in M63 medium supplemented with 10 mM 4-HPA as the sole carbon source or left untreated. Group A includes strains PAK, PAK(*aph*), and PAK(*hpaA*)/pWC011 in M63 medium with 4-HPA. Group B includes strains PAK, PAK(*aph*), and PAK(*hpaA*)/pWC011 in M63 medium without 4-HPA and also strains PAK(*hpaA*) and PAK(*hpaA*)/WC014 in M63 medium with and without 4-HPA.

detected, suggesting that *P. aeruginosa* is not able to utilize 3-HPA as the sole carbon source.

When 3-HPA was used as an inducer, no expression of the *hpaA*::*lacZ* and *aph*::*lacZ* fusions was observed (Fig. 3). Furthermore, when 3-HPA was used in double-disk diffusion assays (as shown in Fig. 4), no noticeable effect was observed compared to that seen with 4-HPA, suggesting that the induction effect of 4-HPA is highly specific (possibly through a specific interaction between 4-HPA molecules and HpaA protein) (see Discussion).

The presence of an *hpaA-aph* locus and induction of neomycin resistance in various *P. aeruginosa* isolates. We wanted to test whether the *aph* gene is commonly located behind the *hpaA* gene on the chromosome of various *P. aeruginosa* isolates. A PCR approach was employed to survey clinical and environmental isolates. Specific primers for a 1-kb DNA fragment encompassing the whole *hpaA* coding region and part of the *aph* coding region were targeted for PCR amplifications to determine the presence of the *hpaA-aph* gene cluster. Strain PAK was included as a positive control, while *E. coli* O157 and water were used as negative controls. From all 58 isolates, including 19 CF isolates, 20 non-CF isolates, and 19 environmental isolates, 1-kb fragments were amplified (data not shown). Furthermore, 56 of these strains were capable of utilizing 4-HPA, as determined by growth on M63 medium supplemented with 4-HPA as a sole carbon source. These results suggest that the coexistence of *hpaA* and *aph* is a common feature in *P. aeruginosa*.

Out of the 58 *P. aeruginosa* isolates, 48 exhibited higher levels of resistance to neomycin than the PAK strain (for which the neomycin MIC is ca. 10 μg/ml in standard MHB medium) and the other 10 showed a level of resistance similar to or even lower than that of strain PAK. Utilizing the double-disk diffusion assay, we further assessed the ability of 4-HPA to induce *aph* expression in these 58 *P. aeruginosa* isolates. A total of 45 strains with various levels of resistance to neomycin were tested; the others (mostly CF isolates) were excluded due to their apparent growth defect on M63 plates or to extremely high resistance to neomycin. Out of the 27 clinical isolates, 19 (including 5 CF and 14 non-CF isolates) showed induction of resistance in the presence of 4-HPA. However, only 3 out of 18 environmental isolates responded positively to the presence of 4-HPA. Thus, a significantly ($P = 6.5864 \times 10^{-5}$) higher portion of the clinical isolates than environmental isolates showed 4-HPA-induced resistance, implying that 4-HPA-mediated induction of *aph* gene plays a role in the life of clinical *P. aeruginosa* isolates, possibly by providing a selection advantage in the challenge of repetitive antibiotic chemotherapy.

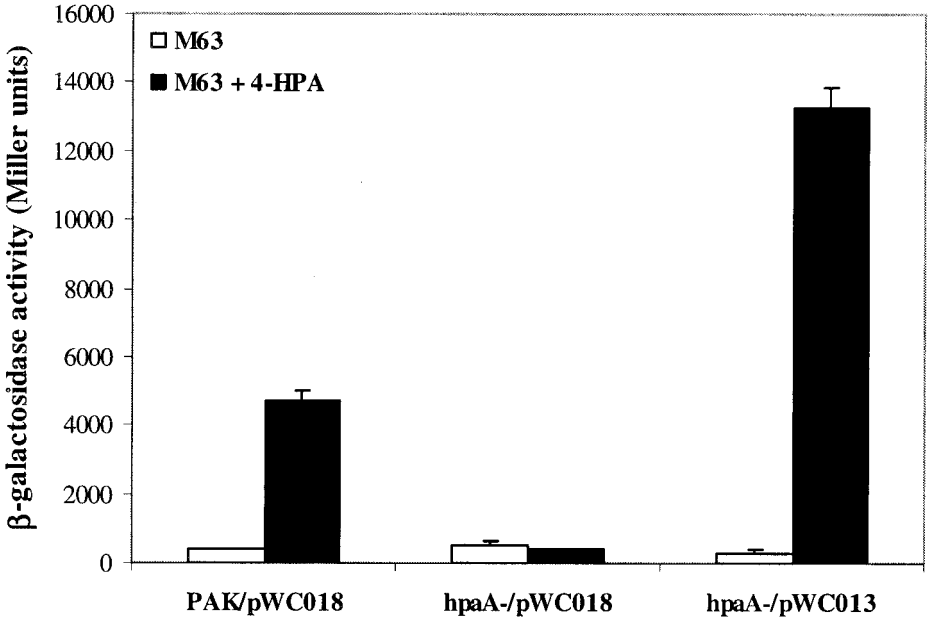

FIG. 6. Expression of PA4121 is under the positive regulation of HpaA in response to the presence of 4-HPA. Cells were grown in M63 medium with (filled bars) or without (empty bars) 5 mM 4-HPA, and β-galactosidase activities were measured. pWC013 and pWC018 are both *lacZ* fusion plasmids for gene PA4121, and pWC013 contains an intact copy of *hpaA* (Fig. 1B).

DISCUSSION

Aminoglycoside-phosphotransferase APH(3′)-IIb of *P. aeruginosa* was shown by Hächler et al. to specifically confer resistance to a group of aminoglycoside antibiotics, including kanamycin A and B, neomycin B and C, butirosin, and seldomycin F5 (9). However, Hächler et al. were not able to use its putative promoter to express the *aph* gene in *E. coli* and no *P. aeruginosa* expression data were presented. Using a genetic approach, we showed that the *aph(3′)-IIb* gene of *P. aeruginosa* strain PAK is positively controlled by its upstream AraC-type regulator HpaA. The *hpaA* promoter drives transcription of *aph*, and this transcription is auto-regulated by HpaA in response to the presence of 4-HPA. Also, aminoglycoside resistance in strain PAK is clearly inducible by 4-HPA in an HpaA-dependent manner. More significantly, the coexistence of *hpaA* and *aph* in *P. aeruginosa* is not limited to strains PAK and PAO1; instead, it is a common feature in both clinical and environmental isolates of *P. aeruginosa*. By showing the growth of strain PAK in 4-HPA-based medium, we demonstrated the ability of *P. aeruginosa* to utilize 4-HPA as a sole carbon source in an HpaA-dependent manner. 4-HPA is actually a better carbon source than glucose or glycerol (data not shown). Therefore, *hpaA-aph* could be an important operon for *P. aeruginosa*, not only conferring *P. aeruginosa* resistance to some of the important aminoglycoside antibiotics (e.g., neomycin and kanamycin) but also enabling it to adjust this resistance according to certain environmental signals, especially in vivo (when *P. aeruginosa* is infecting human or animal hosts), for *P. aeruginosa* has been shown to utilize aromatic compounds found in mammalian nervous system through an *hpa* pathway (6, 21).

Data obtained from 3-HPA in this study were also quite interesting. As a 4-HPA analogue, 3-HPA was suggested to be degraded by the same metabolic pathway as 4-HPA in *E. coli* but by different pathways in fluorescent pseudomonads and *Acinetobacter* spp. (4, 28). However, *P. aeruginosa* strain PAK cannot utilize 3-HPA and it has no significant inducing effect on *hpaA-aph* expression either. Further study is needed to clarify this issue.

A discrepancy has been noticed among the results of the *hpaA-aph* PCR survey (positive results for all 58 isolates), the 4-HPA utilization test (56 positive results out of 58), and the double-disk diffusion assay (22 isolates responsive out of 45). This can be tentatively explained by the following possible factors: (i) mutations in the *hpaA-aph* region, which make this operon either nonfunctional or constitutive; (ii) bacterial defect in the uptake of the 4-HPA; and (iii) other mechanisms of aminoglycoside resistance that mask the *aph*-mediated resistance. For example, alterations in the antibiotic targets likely mask the effect of *aph* induction by 4-HPA.

It has been suggested that *E. coli* W might have acquired its *hpa* catabolic cassette horizontally from other organisms, as evidenced by the fact that homologues flanking the *hpa* regulon are found in *E. coli* K-12, which does not have the *hpa* regulon (19). We have looked into all other organisms with available genomic sequence data and compared the *P. aeruginosa hpa* regulon with its counterparts in these organisms (Fig. 2). The fact that the two *P. aeruginosa hpa* operons are farther separated than their counterparts in *E. coli* W (and that they possess two activators instead of a repressor and an activator) distinguishes *P. aeruginosa* from most other *hpa*-containing bacteria, including *E. coli*, *Salmonella enterica* serovar Typhi, and *S. enterica* serovar Typhimurium, whose *hpa* regulons have both *hpaA* and *hpaR* homologues.

Although β-lactamase genes are almost ubiquitously present on the chromosomes of enterobacteria, few are in salmonellae,

most of which are plasmid borne (2, 13). On the chromosome of *S. enterica* serovar Typhimurium LT2, however, an ORF (no. 1109) encoding a probable β-lactamase is found localized downstream of the *hpaA* homologue, with a 14-bp intergenic region (15), resembling the *hpaA-aph* structure in *P. aeruginosa*. The same structure is also found in the *S. enterica* serovar Typhi CT18 chromosome, in which an almost identical ORF 1143, sharing 99% amino acid identity with ORF 1109 in *S. enterica* serovar Typhimurium, was located immediately downstream to the *hpaA* homologue (17) (Fig. 2). Therefore, it is possible that in salmonellae, the chromosomal β-lactamase (in similarity to that of *P. aeruginosa*) is inducible only under certain environmental conditions. The presence of the probable β-lactamase ORF behind the *hpaA* homologous gene may prove an interesting lead for the study of the possible correlation of *hpa* pathways and inducible antibiotic resistance in these bacteria.

Antibiotic-modifying enzymatic genes have been suggested to have evolved through two different pathways, either being acquired from antibiotic-producing microorganisms that need to defend against their own metabolic by-products or originating from normal metabolic genes and having undergone series of mutations (26). The *aac(6′)-Ic* gene of *Serratia marcescens* is found in all *S. marcescens* strains, while its expression is silent in the aminoglycoside-susceptible ones (27). This gene was suggested to have evolved from a normal metabolic gene, although no physiological evidence is yet available (26). Meanwhile, *E. coli* W has been found to contain (near the *hpa* regulon) a *pac* gene encoding a penicillin G acylase, which is believed to hydrolyze esters of 4-HPA and phenylacetic acids, therefore expanding the substrate spectrum of the *hpa* pathway (22, 23). Although our data indicate that *aph* is not required for the utilization of 4-HPA in *P. aeruginosa*, we certainly have not tested all the possible substrates suitable for the *hpa* pathway; also, our approach would probably have been incapable of detecting any effect of *aph* mutation if the APH were only to play a compensatory role in the *hpa* pathway.

Aminoglycoside-modifying enzyme genes have not been found to be generally regulated. So far, only two of them, the *aac(6′)-Ic* gene of *Serratia marcescens* and the *aac(2′)-Ia* gene of *Providencia stuartii*, are known to be under regulation, and the exact regulatory factor(s) is yet to be discovered (26, 27). Our study of *hpaA-aph* provides a novel regulation model for aminoglycoside-modifying genes: a surrogate type of activator is recruited from another regulatory pathway. This also raised a possible mechanism for the bacteria to acquire increased antibiotic resistance through mutation: mutations within the *hpaA* coding region or its promoter region which cause constitutive activation of *hpaA* by affecting either HpaA-(4-HPA) interaction or HpaA-DNA interaction. It has been mentioned by Hächler et al. that since they found that all 10 tested strains had *aph* genes present, *aph* is likely a ubiquitous gene in *P. aeruginosa* (9). Our study suggests that not just the presence of *aph* alone but also the coexistence of *hpaA* and *aph* seems to be a common feature in *P. aeruginosa*; more importantly, a higher proportion of clinical strains than environmental isolates showed increased neomycin resistance in response to the presence of 4-HPA. We postulate that at least some of the clinical strains might have acquired elevated aminoglycoside resistance through an *hpaA-aph* induction pathway.

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REFERENCES

1. **Bodey, G. P., R. Bolivar, V. Fainstein, and L. Jadeja.** 1983. Infections caused by *Pseudomonas aeruginosa.* Rev. Infect. Dis. **5:**279–313.
2. **Bradford, P. A.** 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. **14:**933–951.
3. **Burlingame, R., and P. J. Chapman.** 1983. Catabolism of phenylpropionic acid and its 3-hydroxy derivative by *Escherichia coli.* J. Bacteriol. **155:**113–121.
4. **Cooper, R. A., and M. A. Skinner.** 1980. Catabolism of 3- and 4-hydroxyphenylacetate by the 3,4-dihydroxyphenylacetate pathway in *Escherichia coli.* J. Bacteriol. **143:**302–306.
5. **Cuskey, S. M., and R. H. Olsen.** 1988. Catabolism of aromatic biogenic amines by *Pseudomonas aeruginosa* PAO1 via *meta* cleavage of homoprotocatechuic acid. J. Bacteriol. **170:**393–399.
6. **Cuskey, S. M., V. Peccoraro, and R. H. Olsen.** 1987. Initial catabolism of aromatic biogenic amines by *Pseudomonas aeruginosa* PAO: pathway description, mapping of mutations, and cloning of essential genes. J. Bacteriol. **169:**2398–2404.
7. **Galimand, M., T. Lambert, G. Gerbaud, and P. Courvalin.** 1993. Characterization of the *aac(6′)-Ib* gene encoding an aminoglycoside 6′-*N*-acetyltransferase in *Pseudomonas aeruginosa* BM2656. Antimicrob. Agents Chemother. **37:**1456–1462.
8. **Govan, J. R., and V. Deretic.** 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia.* Microbiol. Rev. **60:**539–574.
9. **Hächler, H., P. Santanam, and F. H. Kayser.** 1996. Sequence and characterization of a novel chromosomal aminoglycoside phosphotransferase gene, *aph(3′)-IIb*, in *Pseudomonas aeruginosa.* Antimicrob. Agents Chemother. **40:**1254–1256.
10. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77–86.
11. **Kominos, S. D., C. E. Copeland, B. Grosiak, and B. Postic.** 1972. Introduction of *Pseudomonas aeruginosa* into a hospital via vegetables. Appl. Microbiol. **24:**567–570.
12. **Kucera, M., and O. Lysenko.** 1968. The mechanism of pathogenicity of *Pseudomonas aeruginosa.* V. Isolation and properties of the proteinases toxic for larvae of the greater wax moth *Galleria mellonella* L. Folia Microbiol. **13:**288–294.
13. **Livermore, D. M.** 1995. β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8:**557–584.
14. **Mahajan-Miklos, S., M. W. Tan, L. G. Rahme, and F. M. Ausubel.** 1999. Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model. Cell **96:**47–56.
15. **McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson.** 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature **413:**852–856.
16. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Parkhill, J., G. Dougan, K. D. James, N. R. Thomson, D. Pickard, J. Wain, C. Churcher, K. L. Mungall, S. D. Bentley, M. T. Holden, M. Sebaihia, S. Baker, D. Basham, K. Brooks, T. Chillingworth, P. Connerton, A. Cronin, P. Davis, R. M. Davies, L. Dowd, N. White, J. Farrar, T. Feltwell, N. Hamlin, A. Haque, T. T. Hien, S. Holroyd, K. Jagels, A. Krogh, T. S. Larsen, S. Leather, S. Moule, P. O'Gaora, C. Parry, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell.** 2001. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature **413:**848–852.
18. **Poirel, L., T. Lambert, S. Türkoglü, E. Ronco, J.-L. Gaillard, and P. Nordmann.** 2001. Characterization of class 1 integrons from *Pseudomonas aeruginosa* that contain the bla_{VIM-2} carbapenem-hydrolyzing β-lactamase gene and of two novel aminoglycoside resistance gene cassettes. Antimicrob. Agents Chemother. **45:**546–552.
19. **Prieto, M. A., E. Diaz, and J. L. Garcia.** 1996. Molecular characterization of the 4-hydroxyphenylacetate catabolic pathway of *Escherichia coli* W: engineering a mobile aromatic degradative cluster. J. Bacteriol. **178:**111–120.
20. **Prieto, M. A., and J. L. Garcia.** 1997. Identification of a novel positive regulator of the 4-hydroxyphenylacetate catabolic pathway of *Escherichia coli.* Biochem. Biophys. Res. Commun. **232:**759–765.
21. **Prieto, M. A., and J. L. Garcia.** 1997. Identification of the 4-hydroxyphen-

ylacetate transport gene of *Escherichia coli* W: construction of a highly sensitive cellular biosensor. FEBS Lett. **414:**293–297.

22. **Prieto, M. A., and J. L. Garcia.** 1994. Molecular characterization of 4-hydroxyphenylacetate 3-hydroxylase of *Escherichia coli.* A two-protein component enzyme. J. Biol. Chem. **269:**22823–22829.
23. **Prieto, M. A., A. Perez-Aranda, and J. L. Garcia.** 1993. Characterization of an *Escherichia coli* aromatic hydroxylase with a broad substrate range. J. Bacteriol. **175:**2162–2167.
24. **Schwocho, L. R., C. P. Schaffner, G. H. Miller, R. S. Hare, and K. J. Shaw.** 1995. Cloning and characterization of a 3-*N*-aminoglycoside acetyltransferase gene, *aac(3)-Ib*, from *Pseudomonas aeruginosa.* Antimicrob. Agents Chemother. **39:**1790–1796.
25. **Shaw, K. J., H. Munayyer, P. N. Rather, R. S. Hare, and G. H. Miller.** 1993. Nucleotide sequence analysis and DNA hybridization studies of the *ant*(*4′*)-*IIa* gene from *Pseudomonas aeruginosa.* Antimicrob. Agents Chemother. **37:**708–714.
26. **Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller.** 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Rev. **57:**138–163.
27. **Shaw, K. J., P. N. Rather, F. J. Sabatelli, P. Mann, H. Munayyer, R. Mierzwa, G. L. Petrikkos, R. S. Hare, G. H. Miller, P. Bennett, et al.** 1992. Characterization of the chromosomal *aac*(*6′*)-*Ic* gene from *Serratia marcescens.* Antimicrob. Agents Chemother. **36:**1447–1455.
28. **Sparnins, V. L., P. J. Chapman, and S. Dagley.** 1974. Bacterial degradation of 4-hydroxyphenylacetic acid and homoprotocatechuic acid. J. Bacteriol. **120:**159–167.
29. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory, and M. V. Olson.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406:**959–964.
30. **Totten, P. A., and S. Lory.** 1990. Characterization of the type a flagellin gene from *Pseudomonas aeruginosa* PAK. J. Bacteriol. **172:**7188–7199.
31. **Wang, Y., U. Ha, L. Zeng, and S. Jin.** 2003. Regulation of membrane permeability by a two-component regulatory system in *Pseudomonas aeruginosa.* Antimicrob. Agents Chemother. **47:**95–101.
32. **West, S. E., H. P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky.** 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa.* Gene **148:**81–86.