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NOTES

Sequence and Characterization of a Novel Chromosomal Aminoglycoside Phosphotransferase Gene, *aph(3′)-IIb*, in *Pseudomonas aeruginosa*

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A novel, probably chromosomally encoded, aminoglycoside phosphotransferase gene was cloned on a 2,996-bp *Pst*I fragment from *Pseudomonas aeruginosa* and designated *aph(3′)-IIb*. It coded for a protein of 268 amino acids that showed 51.7% amino acid identity with APH(3′)-II [APH(3′) is aminoglycoside-3′-phosphotransferase] from Tn*5*. Two other open reading frames on the cloned fragment showed homology to a signal-transducing system in *P. aeruginosa*.

Three classes of enzymes, aminoglycoside acetyl-, nucleotidyl-, and phosphotransferases (AAC, ANT, and APH, respectively) mediate bacterial resistance to aminoglycosides by modification of the drugs (14). Recently, ANT(4′)-IIa was discovered in a clinical isolate, *Pseudomonas aeruginosa* 005, harboring plasmid pMG77 (7), and the gene encoding ANT(4′)-IIa was characterized and sequenced (13). The present study describes an additional aminoglycoside resistance gene cloned from *P. aeruginosa* 005.

Bacterial strains (Table 1) were grown in 2× YT broth or on 2× YT agar (12). Antibiotic susceptibilities were evaluated by standard procedures of the National Committee for Clinical Laboratory Standards (11). Aminoglycoside-modifying enzyme activities in osmotic shock extracts and aminoglycoside inactivation by bioassay were performed as described previously (7). Whole-cell and plasmid DNA preparations, DNA manipulations, agarose gel electrophoresis, and Southern hybridization were done by following standard protocols (12). α-^{32}P-labeled probes were prepared by using a random primed DNA labeling kit (Boehringer, Mannheim, Germany). Resistance determinants were cloned by ligation of *Pst*I-digested vector (pTZ18R) and insert DNA (*P. aeruginosa* SCH84043005); this was followed by transformation into *Escherichia coli* DH5α by the $CaCl_2$ method (12). Transformants were selected on 2× YT agar containing 25 μg of kanamycin per ml. Sequencing of appropriate subclones (Fig. 1) in M13mp18/19 was done with a Sequenase kit (United States Biochemical, Cleveland, Ohio). The sequence was deposited in the EMBL Data Bank. Sequences were analyzed by using the DNA Inspector IIe (Textco, West Lebanon, N.H.) and the GCG Sequence Analysis Software Package, version 7.3 (Genetics Computer Group, Madison, Wis.).

Selection with tobramycin while cloning aminoglycoside resistance from *P. aeruginosa* SCH84043005 yielded the *ant(4′)-IIa* gene (7) in pSHA1. Selection with kanamycin led to an additional clone, pSHB1, that carried a 3-kb *Pst*I fragment (Table 1; Fig. 1). *E. coli* DH5α harboring pSHB1 was resistant to kanamycin A (MIC, >128 μg/ml), kanamycin B (MIC, 64 μg/ml), neomycin B and C (MIC, 8 μg/ml), butirosin (MIC, 8 μg/ml), and seldomycin F5 (MIC, >128 μg/ml), but it was susceptible to lividomycin A (MIC, 2 μg/ml), lividomycin B (MIC, 1 μg/ml), gentamicin (MIC, 0.125 μg/ml), tobramycin (MIC, 0.125 μg/ml), amikacin (MIC, 0.25 μg/ml), and dibekacin (MIC, 0.125 μg/ml). A plasmid with the 3-kb fragment in the opposite orientation mediated no resistance, implicating that a vector promoter was responsible for expression. The enzyme produced by *E. coli* DH5α(pSHB1) phosphorylated and inactivated kanamycin B, butirosin, neomycin B, and seldomycin F5. A subcloned 1.2-kb *Pst*I-*Bam*HI fragment was functional only in the same orientation as that in pSHB1. The APH(3′) phenotype could not be detected in the wild-type strain SCH84043005 since it was masked by the ANT(4′)-IIa which encompassed the substrate spectrum of the former enzyme (7). A Southern blot with total DNA of the *P. aeruginosa* strains SCH84043005 (donor), PAO38 Rif^r (recipient), and PAO38 Rif^r(pMG77) (transconjugant), all of which were cleaved with *Pst*I or *Pst*I-*Bam*HI, was probed with labeled pSHB1. All three strains showed identical hybridization signals at 3, 1.2, and 1.8 kb, respectively. This indicated that the cloned 3-kb fragment was not linked to plasmid pMG77 but was located either on the chromosome or on an unknown large plasmid present in the original SCH84043005 strain and the PAO38 Rif^r recipient strain. Hybridization, under conditions identical to those described above, of all of 10 additional *P. aeruginosa* strains selected from 104 clinical isolates screened for their kanamycin MICs (MIC range, 4 to ≥256 μg/ml), including ATCC 27853, showed one distinct hybridizing band. In 7 of the 10 strains, this was a 3-kb band, as in SCH84043005. The sequence of the 3-kb fragment had 2,996 bp. An open reading frame (ORF) with a coding capacity of 268 amino acids was identified as an aminoglycoside phosphotransferase gene (*aph*) by homology (Fig. 2). Putative binding sites for the ribosome (AGGAAG) and for the transcription complex (−10, TATACT; −35, TCGCTG) were identified at 15, 38, or 58 bp upstream of the translational start site, respectively. However, they were not functional in

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TABLE 1. Bacterial strains and plasmids[a]

Strain, plasmid, or phage	Relevant properties	Reference or source
P. aeruginosa		
005[b]	Clinical isolate; Km^r (MIC, 256 μg/ml), Ak^r, and Tm^r by *ant(4′)-IIa* on pMG77	7
PAO38 Rif^r	Recipient for pMG77 by conjugation; FP^- *leu*, Km^i (MIC, 32 μg/ml), Rif^r	7
PAO38 (pMG77) Rif^r	Transconjugant SCH84043005 × PAO38 Rif^r, Km^r (MIC, 256 μg/ml), Ak^r, Tm^r	7
PASH1	Clinical isolate; Km^r (MIC, 128 μg/ml)	This report
PASH2	Clinical isolate; Km^r (MIC, 128 μg/ml)	This report
PASH3	Clinical isolate; Km^i (MIC, 32 μg/ml)	This report
PASH4	Clinical isolate; Km^s (MIC, 16 μg/ml)	This report
PASH5	Clinical isolate; Km^s (MIC, 16 μg/ml)	This report
PASH6	Clinical isolate; Km^i (MIC, 32 μg/ml)	This report
PASH7	Clinical isolate; Km^i (MIC, 32 μg/ml)	This report
PASH8	Clinical isolate; Km^s (MIC, 16 μg/ml)	This report
PASH9	Clinical isolate; Km^s (MIC, 4 μg/ml)	This report
ATCC 27853	Km^r (MIC, 256 μg/ml)	ATCC
E. coli DH5α	Standard host for recombinant plasmids (12)	Bethesda Research Laboratories
plasmid or phage		
pTZ18R	Multicopy cloning vector	United States Biochemical
M13mp18/19	Phage M13-based sequencing vector	Boehringer Mannheim
pSHA1	pTZ18R with cloned *ant(4′)-IIa* on a 2.8-kb *Pst*I fragment from SCH84043005	This work and 7
pSHB1	pTZ18R with cloned *aph* gene on a 3-kb *Pst*I fragment from SCH84043005	This work

[a] Abbreviations: Ak^r, resistance to amikacin; Km^i, intermediate resistance to kanamycin; Km^s, susceptibility to kanamycin; Rif^r, resistance to rifampin; Tm^r, resistance to tobramycin; ATCC, American Type Culture Collection.
[b] The full name is *P. aeruginosa* SCH84043005 (6).

E. coli. The APH(3′) in *P. aeruginosa* was most closely related to APH(3′)-IIa on Tn*5* (51.7% amino acid identity and 66.5% similarity) (Fig. 2) and was designated APH(3′)-IIb. All conserved amino acids within the three functionally important motifs near the carboxy terminus (9) were identical in APH(3′)-IIb. Additionally, most conserved amino acids upstream of these motifs (1, 2, 9, 15, 16) were also found in APH(3′)-IIb, the only two exceptions being the changes of amino acid Y or F at position 37 (Y/F_{37})→H, and E_{161}→D (numbering according to Blazquez et al. [1]). Moreover, Y_{218}, which is known to be critical for a low K_m value for the wild-type APH(3′)-II (8), is changed to H in APH(3′)-IIb.

Translations of most additional ORFs in the cloned 3-kb fragment (ORFs 238, 186, 154, 153, and 131) (Fig. 1) did not show significant homology to entries in protein databases. One-third of ORF 189 showed 25.8% identity (50% similarity) with a GTP binding protein from *Arabidopsis thaliana*, and 90% of ORF 239 was 31.9% identical (57.8% similar) to the signal sensor PilS, which phosphorylates the response regulator PilR in *P. aeruginosa* (5). These homologies led to the speculation that the genes for an unknown regulator or signal transducer system might be located near the *aph(3′)-IIb* locus.

Although APH(3′)-II activity has been reported in *P. aeruginosa* since 1975 (10), it was found rarely in clinical isolates (14),

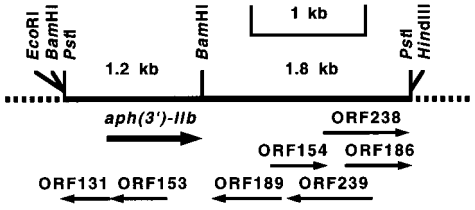

FIG. 1. Physical map of pSHB1 showing the *aph(3′)-IIb* gene (bold arrow) and the additional ORFs with potential coding capacities of >100 amino acids (plain arrows). Bold line, 3-kb cloned DNA; dashed line, pTZ18R vector DNA.

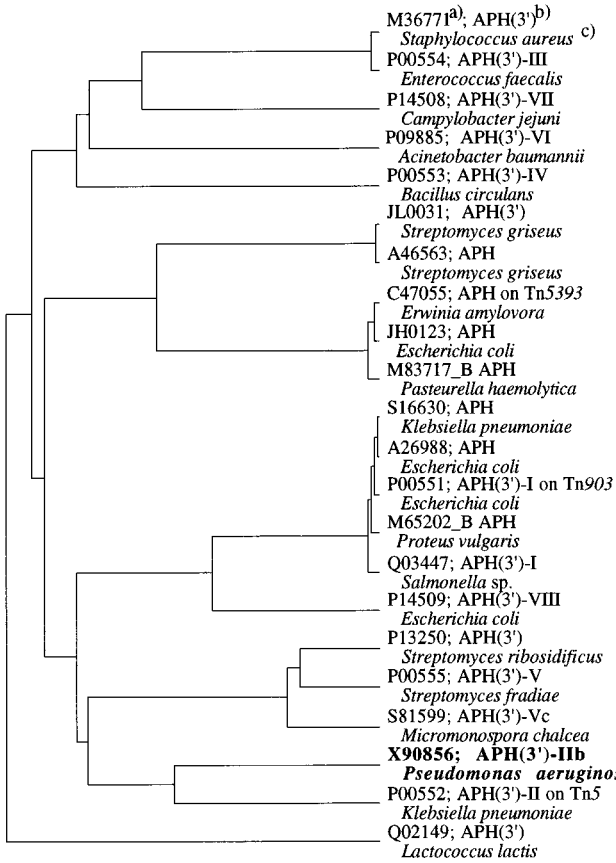

FIG. 2. Clustering of the relative pairwise similarities of 22 APH(3′) enzymes from 17 species as drawn by the GCG program Pileup. APH(3′)-IIb is shown in boldface type. a), Mipsx-, PIR-, or SwissProt. accession numbers; b), aminoglycoside-3′-phosphotransferase; c), original host.

and a corresponding gene was identified in only three of six such strains by using an internal probe prepared from *aph(3′)-IIa* on Tn*5* (17). No gene encoding an APH(3′) from *P. aeruginosa* has so far been cloned or sequenced. The present report provides the sequence of *aph(3′)-IIb*, which is probably chromosomally located, from *P. aeruginosa* 005. The gene was found in all nine clinical isolates tested and also in the National Committee for Clinical Laboratory Standards reference strain ATCC 27853 and likely accounts at least partly for the "uniform resistance" (4) of *P. aeruginosa* to kanamycin. The ubiquity of *aph(3′)-IIb* in *P. aeruginosa* suggests that it may be a normal metabolic gene, supporting the hypothesis that modification of the regulation of metabolic genes may generate resistance to aminoglycosides (14). This hypothesis is also supported indirectly by a report describing an APH homolog unable to confer aminoglycoside resistance within the *his* cluster of *Lactococcus lactis* (3). Because of the low level of homology between *aph (3′)-IIb* and *aph(3′)-IIa* from Tn*5* (56.8% at the DNA level), the two genes are unlikely to hybridize under usually applied conditions. Therefore, the strains of *P. aeruginosa* reported to express an APH(3′)-II phenotype, despite a lack of hybridization to *aph(3′)-IIa* (17), might be explained by an elevated level of expression of *aph(3′)-IIb*, which is widespread in this organism. The substrate spectrum of the novel APH(3′)-IIb enzyme from *P. aeruginosa* concurs with those of other APH(3′)-II enzymes (14), showing that functionally equivalent aminoglycoside-modifying enzymes can be structurally diverse.

Nucleotide sequence accession number. The sequence of *aph(3′)-IIb* determined in the present study was deposited in EMBL Data Bank under accession number X90856.

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