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Isolation, Characterization, and DNA Sequence Analysis of an AAC(6′)-II Gene from *Pseudomonas aeruginosa*

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The gene encoding a 6′-*N*-acetyltransferase, AAC(6′)-II, was cloned from *Pseudomonas aeruginosa* plasmid pSCH884. This gene mediates resistance to gentamicin, tobramycin, and netilmicin but not amikacin or isepamicin. The DNA sequence of the gene and flanking regions was determined. The 5′- and 3′-flanking sequences showed near identity to sequences found abutting a variety of different genes encoding resistance determinants. It is likely that the current structure arose by the integration of the 572-base-pair sequence containing the AAC(6′)-II gene into a Tn*21*-related sequence at the recombinational hot spot, AAAGTT. We have compared the sequence of the AAC(6′)-II gene to genes of other 6′-*N*-acetyltransferases. An AAC(6′)-Ib protein (encoded by the *aacA4* gene; G. Tran Van Nhieu and E. Collatz, J. Bacteriol. 169:5708–5714, 1987) that results in resistance to amikacin but not gentamicin was found to share 82% sequence similarity with the AAC(6′)-II protein. We speculate that these two genes arose from a common ancestor and that the processes of selection and dissemination have led to the observed differences in the spectrum of aminoglycoside resistance.

Bacteria produce three types of enzymes that are capable of modifying aminoglycosides. The clinical observation of high levels of aminoglycoside resistance is often due to enzymatic inactivation by these acetyltransferases, nucleotidyltransferases, and phosphotransferases (6). The genes for these modifying enzymes are usually plasmid borne (6) and are often found inserted into transposons (14, 25).

Four classes of aminoglycoside-acetylating enzymes have been characterized, based on the position of the modified amino group of 2-deoxystreptamine-derived aminoglycosides: AAC(1), AAC(2′), AAC(3), and AAC(6′) (16, 24). High-pressure liquid chromatographic (HPLC) analysis has demonstrated that the acetylated products of these four reactions are distinct (15, 16). In addition, aminoglycoside resistance profiles can be used to identify the presence of each of these enzymes in a host strain (16, 22).

The 6′-*N*-acetyltransferases are of particular epidemiological interest due to the potential for modification of the clinically important aminoglycosides gentamicin, tobramycin, netilmicin, amikacin, and sisomicin. Two subclasses have been distinguished by aminoglycoside resistance profiles: AAC(6′)-I enzymes, which typically modify amikacin; and AAC(6′)-II enzymes, which typically modify gentamicin (22). AAC(6′)-I enzymes have alternatively been called AAC(6′)-IV (24) or AAC(6′)-4 (33), whereas AAC(6′)-II-enzymes have alternatively been called AAC(6′)-III (24).

Several genes encoding 6′-*N*-acetyltransferase type I enzymes have been cloned. The *aacA1* gene from *Citrobacter diversus* encodes a protein of 185 amino acids [AAC(6′)-Ia] and has flanking DNA sequences related to Tn*21* (31). The *aacA4* gene was cloned from an IncM plasmid isolated from *Serratia marcescens* (33). Six amino acids of the β-lactamase signal peptide were found to be fused to this AAC(6′)-Ib protein. A chromosomal gene that encodes an AAC(6′) enzyme has also been cloned from *S. marcescens* [AAC(6′)-Ic] (4).

In clinically isolated *Pseudomonas* strains, one of the most prevalent forms of aminoglycoside resistance is associated with an AAC(6′)-II enzyme (22, 29). In this study we have isolated plasmid pSCH884 from *Pseudomonas aeruginosa* SCH80090884 and shown that it encodes resistance to gentamicin, tobramycin, and netilmicin. We have found that the resistance profiles of this strain and *Escherichia coli* transformants containing the subcloned gene were consistent with AAC(6′)-II activity. In addition, HPLC analysis of the reaction products of extracts of these strains demonstrated the conversion of sisomicin to the 6′-*N*-acetylated product. Physical mapping of gamma-delta insertion mutations and DNA sequence analysis has allowed the prediction of the amino acid sequence of the AAC(6′)-II protein.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used in this study are listed in Table 1. SCH 80090884 is a strain of *P. aeruginosa* isolated in 1980 at The Tokyo Clinical Research Center, Tokyo, Japan. *E. coli* MG1063 was kindly provided by C. M. Berg. RSF1010, pKT212, and PAO38, used for cloning experiments in *Pseudomonas* species, were a gift from G. Jacoby. pMT11s was kindly provided by H. Huang and K. Moore. pIBI30 is a commercially available vector (International Biotechnologies, Inc. [IBI], New Haven, Conn.).

Spontaneous streptomycin-resistant derivatives of *E. coli* C600 and W3110 were selected by plating approximately 10^{10} cells on agar containing 100 μg of streptomycin per ml (23).

All *Pseudomonas* strains were grown on unsupplemented Mueller-Hinton medium (Difco Laboratories, Detroit, Mich.); all *E. coli* strains were grown on LB medium (BBL Microbiology Systems, Cockeysville, Md.). Incubations were performed at 37°C.

Bacterial transformation and conjugation. *E. coli* 294 and MG1063 (Table 1) were transformed by the method of Maniatis et al. (17). Competent cells of *P. aeruginosa* were prepared and transformed as described by Mercer and Loutit (20). Antibiotic concentrations for selections were as follows: 100 μg of ampicillin, 10 μg of tobramycin, 30 μg of netilmicin, 300 μg of sulfisoxazole, and 100 μg of streptomy-

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TABLE 1. Bacterial host strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Reference
P. aeruginosa		
PAO38	Str^s	26
SCH80090884	AAC(6')-II: $Gent^r$ Tob^r Net^r	This work
E. coli		
294	*end hsdR thi pro*	2
C600	Str^r	This work
W3110	Str^r	This work
MG1063	F^+ *recA56*	10
Plasmids		
pSCH884	24-kb AAC(6')-II plasmid from SCH80090884	This work
RSF1010	8.3 kb, Str^r Su^r	1
pKT212	15.9 kb, Str^r Tet^r Cm^r	1
pSCH063	6.3-kb *Eco*RI fragment from pSCH884 cloned into RSF1010, AAC(6')-II	This work
pMT11s	2.3-kb derivative of pBR322, πVX polylinker, Amp^r Tet^s	28
pIBI30	2.8 kb, Amp^r	IBI
pSCH1001	6.3-kb *Eco*RI fragment of pSCH884 cloned into pMT11s, AAC(6')-II	This work
pSCH1002	3.5-kb *Eco*RI-*Hin*dIII fragment of pSCH884 cloned into pMT11s, AAC(6')-II	This work
pSCH1003	2.1-kb *Bam*HI-*Hin*dIII fragment of pSCH884 cloned into pMT11s, AAC(6')-II	This work
pSCH1005	564-bp *Rsa*I-*Hin*dIII fragment of pSCH1003 cloned into the *Sma*I site of pIBI30 (orientation I)	This work
pSCH1007	580-bp *Ava*I fragment of pSCH1003 cloned into the *Sma*I site of pIBI30	This work
pSCH1008	564-bp *Rsa*I-*Hin*dIII fragment of pSCH1003 cloned into the *Sma*I site of pIBI30 (orientation II)	This work
pAZ505	AAC(6')-Ib fragment cloned into the *Cla*I site of pBR322	33
pSCH1012	*Pst*I fragment of pKT212 cloned into pAZ505	This work

cin per ml. Bacterial conjugations were performed in liquid medium (34).

Gamma-delta mutagenesis of the AAC(6')-II gene. Gamma-delta (Tn*1000*) is an insertion element found on the *E. coli* fertility factor F (10). This insertion element has been previously utilized for mutagenesis and localization of genes (12, 34). The *recA* F^+ *E. coli* strain MG1063 was transformed with pSCH1002 (Table 1). Plasmid DNA was isolated from individual ampicillin-resistant transformants. The uncut DNA was analyzed by agarose gel electrophoresis to determine whether the transformants contained plasmids in multimeric or monomeric form. A single MG1063 transformant containing a monomeric form of pSCH1002 was mated to a streptomycin-resistant F^- recipient strain (W3110 or C600). Conjugative transfer of the nonconjugative plasmid pSCH 1002 resulted from mobilization of transient F/pSCH1002 cointegrates, whose formation was catalyzed by gamma-delta.

Preparation and analysis of DNA. Plasmid DNA was isolated from *P. aeruginosa* and *E. coli* by alkaline lysis for large-scale preparations and by the boiling method for small-scale preparations (17). Restriction enzyme digestions, ligations, fill-in reactions with the Klenow fragment of polymerase I, and labeling of DNA with T4 DNA kinase were performed according to the instructions of the supplier (New England BioLabs, Beverly, Mass.). Restriction enzyme-generated DNA fragments were analyzed by agarose gel electrophoresis with Tris-borate-EDTA buffer (17).

Nucleotide sequencing. The DNA sequences of both strands of the 506-base-pair (bp) *Ava*I-*Ava*II fragment of pSCH1002 (Fig. 1, Table 1) were determined by the method of Maxam and Gilbert (18). The 349-bp *Dde*I-*Ava*I fragment was sequenced in the same manner.

Two fragments were cloned into pIBI30 for dideoxy sequencing (27). The ends of a 580-bp *Ava*I fragment from pSCH1002 were filled in with the Klenow fragment of polymerase I, and the fragment was ligated into the *Sma*I site of pIBI30, resulting in plasmid pSCH1007 (Table 1). Similarly, a 564-bp *Rsa*I-*Hin*dIII fragment was subcloned into the *Sma*I site of pIBI30, resulting in plasmids pSCH1005 (orientation I) and pSCH1008 (orientation II). The orientations were determined by restriction mapping the position of the *Ava*II site within the insert (Fig. 1, Table 1).

Oligonucleotides homologous to the Sp6 and the T7 promoter regions were used as primers for sequencing pSCH1005, pSCH1007, and pSCH1008. Additional oligonucleotide primers were used to sequence the internal regions of the pSCH1005, pSCH1007, and pSCH1008 clones. Primers were supplied by Research Genetics (Huntsville, Ala.) and American Biotechnologies, Inc. (Cambridge, Mass.). DNA fragments were resolved by electrophoresis on 6, 8, and 20% polyacrylamide gels containing 8% urea (17). The gels were autoradiographed with XAR5 film (Eastman Kodak Co., Rochester, N.Y.).

Enzymes and biochemicals. T4 DNA ligase, the Klenow fragment of polymerase I, T4 DNA kinase, and all restriction enzymes were obtained from New England BioLabs. Calf intestinal phosphatase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). The Sequenase kit was obtained from U.S. Biochemical Corp. (Cleveland, Ohio). All radiolabeled compounds were obtained from Amersham Corp. (Arlington Heights, Ill.).

All biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.), with the following exceptions: tobramycin (Eli Lilly & Co., Indianapolis, Ind.); amikacin (Bristol Laboratories, Syracuse, N.Y.); gentamicin, netilmicin, 2'-*N*-ethylnetilmicin, 6'-*N*-ethylnetilmicin, and isepamicin (prepared at Schering-Plough Research, Bloomfield, N.J.).

Preparation of extracts for HPLC analysis. Crude enzyme extracts were prepared as described previously (15) with the following modifications. For plasmid maintenance, 2 μg of tobramycin per ml was added to the blood agar plates used to

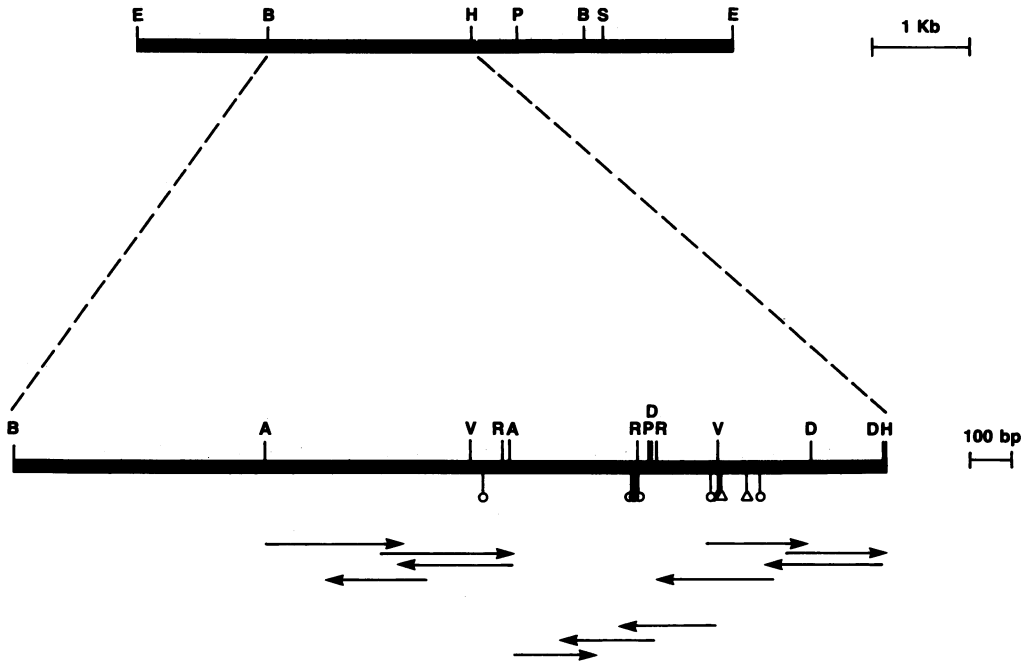


FIG. 1. Restriction map, location of gamma-delta insertion mutations, and sequencing strategy for the AAC(6')-II gene. The 6.3-kb *Eco*RI fragment of pSCH884 (top line). The 2.1-kb *Bam*HI-*Hin*dIII fragment was subcloned into pMT11s (bottom line). The relative positions of 10 gamma-delta insertion mutations (2 identical) are indicated as follows: ○, orientation I; △, orientation II. The direction and extent of DNA sequence analysis are indicated by the arrows on the bottom. Restriction sites: A, *Ava*I; V, *Ava*II; B, *Bam*HI; E, *Eco*RI; D, *Dde*I; H, *Hin*dIII; P, *Pvu*I; R, *Rsa*I; S, *Sal*I.

grow the overnight cultures. Acetylation reactions were performed by using 100 μg of sisomicin per ml and 600 μg of acetyl coenzyme A per ml. Reaction mixtures were incubated overnight at 37°C and filtered through 0.22-μm-pore-size cellulose acetate filters (Corning Glass Works, Corning, N.Y.).

HPLC. HPLC was performed with an integrated HP 1090A liquid chromatographic system (Hewlett-Packard Co., Palo Alto, Calif.). Chromatographic separations were obtained by employing a YMC C-18 (5-μm-pore-size), 25-cm by 6.0-mm analytical column (YMC Corp., Morris Plains, N.J.). A small guard column of C-18 (7-μm-pore-size) reverse-phase material (1.5 cm by 3.2 mm; Brownlee Laboratories, Santa Clara, Calif.) preceded the analytical column. The mobile phases used to achieve selectivity were identical to those described by Lovering et al. (15). Gradient and flow conditions were modified as detailed below. Mobile phase A consisted of methanol-water-glacial acetic acid (50:45:5) in 0.0227 M heptane sulfonic acid sodium salt. Mobile phase B contained the same components with a 90:5:5 ratio. Vacuum-degassed and filtered (0.40-μm-pore-size filter) mobile phases were delivered at 1 ml/min. A linear gradient-run program with the following steps was utilized: 70% A to 80% B (15 min), hold (3 min) to 100% B (5 min), hold (5 min), return to initial conditions (2 min). Precolumn reaction of the acetylated aminoglycoside product with *ortho*-phthalaldehyde produced the corresponding isoindole derivative, which allowed UV detection at 330 nm (0.40 atomic units full scale).

***ortho*-Phthalaldehyde precolumn automated derivatization.** A freshly prepared solution of the derivatization reagent was prepared by adding 200 mg of *ortho*-phthalaldehyde in 0.5 ml of methanol to 9.5 ml of 1 M boric acid containing 250 mM thioglycolic acid. The pH of the solution was adjusted to pH 9 with 40% potassium hydroxide. After filtration (0.40-μm-pore-size filter), this reagent was transferred to an autosampler vial. For component analysis, 100 μl of enzymatic sample or aminoglycoside reference substrate was diluted with an equal volume of isopropanol, allowed to mix for 30 s, and then filtered through a 0.40-μm-pore-size membrane. The samples were transferred to autosampling vials and loaded into a magazine rack along with the derivatization reagent vial for programmed derivatization and analysis.

Automated derivatization involved drawing the reagent (40 μl) and sample (80 μl) within the injection loop such that the sample was sandwiched between the reagent. The solutions were then moved at a finite volume in the injection loop over a given number of cycles to produce efficient mixing. After a programmed wait time of 3 min, the derivative sample was injected onto the HPLC column support.

RESULTS

Cloning the resistance determinant. Plasmid DNA was isolated from SCH80090884, and a single 24-kb plasmid (pSCH884) was observed (Table 1). This DNA was used to transform the susceptible *Pseudomonas* host, strain PAO38. The resulting transformants contained the identical 24-kb plasmid and now showed the classical AAC(6')-II resistance profile (resistance to gentamicin, tobramycin, netilmicin, 5-epi-sisomicin, and 2'-*N*-ethylnetilmicin) (Table 2). Vector RSF1010, which carries resistance to sulfisoxazole (1), was used in subcloning experiments. Plasmid pSCH884 was digested with *Eco*RI, which resulted in four fragments (13.4, 6.3, 4.6, and 0.5 kb). The four fragments were ligated into the *Eco*RI site of RSF1010, and the resulting plasmids were used to transform *Pseudomonas* strain PAO38, selecting for sulfisoxazole and/or netilmicin resistance. Netilmicin plus sulfisoxazole resistance was found only in transformants that contained the 6.3-kb *Eco*RI fragment [PAO38(pSCH063); Fig. 1, Table 1].

TABLE 2. Susceptibility of *P. aeruginosa* and *E. coli* strains to selected aminoglycosides

Organism	MIC (μg/ml)[a]							
	GENT	TOB	AMIK	ISEP	NET	5-EPI	2'NET	6'NET
P. aeruginosa								
SCH80090884	256	256	2	4	>256	>256	>512	16
PAO38	1	0.5	2	4	2	0.5	8	16
PAO38(pSCH884)[b]	32	32	2	2	128	128	>512	8
PAO38(pSCH1012)[c]	4	32	16	16	>128	>64	>512	32
E. coli								
C600	1	1	2	0.5	0.5	0.5	1	1
C600(pSCH1001)[d]	4	16	4	0.5	0.5	4	16	0.5
C600(pSCH1003)[e]	4	32	4	0.5	2	2	8	0.5
C600(pSCH1002:9)[f]	2	8	2	0.5	0.5	2	16	2
C600(pSCH1002:4)[g]	1	1	4	0.5	0.5	1	1	1
294	1	≤0.5	≤1	≤1	≤1	≤0.5	≤4	≤4
294(pSCH1012)[c]	1	16	8	2	16	8	64	≤4

[a] MICs were determined in microdilution plates containing 0.1 ml of unsupplemented Mueller-Hinton broth (plus drug). Plates were inoculated at approximately 5×10^4 cells per ml and incubated at 37°C for 24 h. Abbreviations: GENT, gentamicin; TOB, tobramycin; AMIK, amikacin; ISEP, isepamicin; NET, netilmicin; 5-EPI, 5-epi-sisomicin; 2'NET, 2'-*N*-ethylnetilmicin; 6'NET, 6'-*N*-ethylnetilmicin.

[b] A 24-kb plasmid from the original clinical isolate, which carries the AAC(6')-II gene.

[c] AAC(6')-Ib gene on a *P. aeruginosa-E. coli* shuttle vector.

[d] A 6.3-kb *Eco*RI fragment containing the AAC(6')-II gene cloned into pMT11s.

[e] A 2.1-kb *Bam*HI-*Hin*dIII fragment containing the AAC(6')-II gene cloned into pMT11s.

[f] C600(pSCH1002:9) (tobramycin resistant) has gamma-delta inserted into a region outside of the AAC(6')-II gene.

[g] C600(pSCH1002:4) (tobramycin susceptible) has gamma-delta inserted into the coding region of the AAC(6')-II gene.

The 6.3-kb *Eco*RI fragment from pSCH884 was also ligated into the *Eco*RI site of the *E. coli* vector pMT11s (28). *E. coli* transformants were selected by using 10 μg of tobramycin per ml. These resistant transformants carried the resulting plasmid pSCH1001 and showed higher MICs of gentamicin, 5-epi-sisomicin, and 2'-*N*-ethylnetilmicin (Table 2). An increase in MIC was not observed for netilmicin.

Plasmid pSCH1001 had a convenient *Hin*dIII restriction site near the center of the cloned fragment (Fig. 1). We subcloned both the 3.5-kb and the 2.8-kb *Eco*RI-*Hin*dIII fragments into pMT11s that had been cut with *Eco*RI-*Hin*dIII. All of the 11 transformants that contained the 3.5-kb fragment inserted into pMT11s (pSCH1002) were resistant to 10 μg of tobramycin per ml, whereas all of the 15 transformants that contained the 2.8-kb fragment inserted into pMT11s were susceptible to tobramycin. These data suggested that the AAC(6')-II gene was located within the 3.5-kb *Eco*RI-*Hin*dIII fragment of pSCH1002.

Localization of the tobramycin resistance determinant by gamma-delta mutagenesis. The F^+ donor strain MG1063 (pSCH1002) was mated with C600 (Str^r) or W3110 (Str^r). Ampicillin-resistant, streptomycin-resistant transconjugants were selected and patched to plates containing 10 μg of tobramycin, 100 μg of streptomycin, and 100 μg of ampicillin per ml. Of 109 transconjugants tested from both matings, 29 were susceptible to tobramycin but resistant to streptomycin and ampicillin. These tobramycin-susceptible colonies were presumed to contain gamma-delta inserted into the AAC(6')-II gene.

Plasmid DNA isolated from 10 tobramycin-susceptible transconjugants was analyzed. Detailed restriction analysis was performed on each of the transconjugants, as well as the original plasmid pSCH1002, to determine the location of the gamma-delta insertions. All of the insertions mapped within a 700-bp region, which defined the minimum size of this gene and its promoter (Fig. 1).

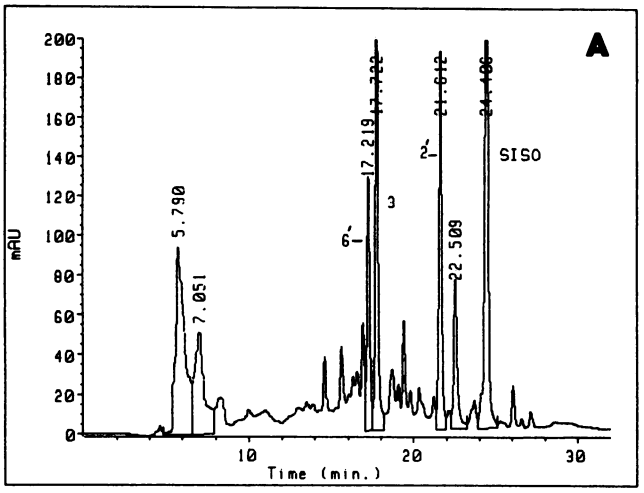
To obtain a smaller clone containing this gene, we subcloned a 2.1-kb *Bam*HI-*Hin*dIII fragment from pSCH1002 into pMT11s, which had been cut with *Bam*HI-*Hin*dIII (Fig. 1). The resulting transformant (pSCH1003) was also resistant to tobramycin.

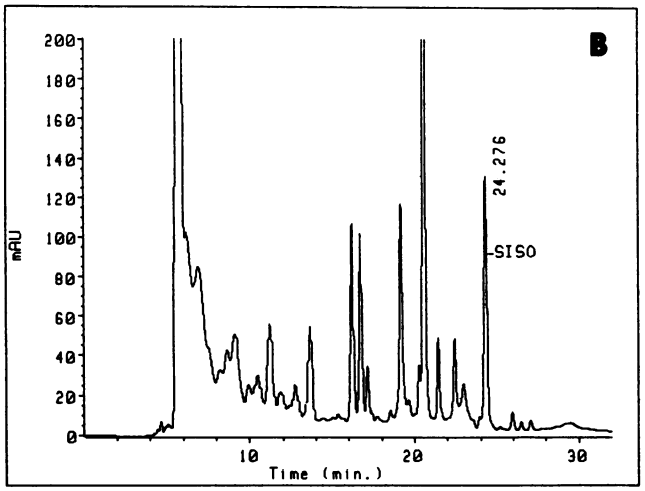
Expression of the AAC(6')-II and AAC(6')-Ib genes in *E. coli* and *P. aeruginosa*. Standard microdilution MIC testing was performed on transformants containing plasmid pSCH884 and its derivatives. The clinically isolated SCH80090884 was resistant to gentamicin, tobramycin, netilmicin, 5-epi-sisomicin, and 2'-*N*-ethylnetilmicin but not 6'-*N*-ethylnetilmicin, amikacin, and isepamicin (Table 2). The large ratio between the MIC of 2'-*N*-ethylnetilmicin and 6'-*N*-ethylnetilmicin was a classic indication of 6'-*N*-acetylating activity (22). When this plasmid was transformed into the susceptible *Pseudomonas* host, PAO38, the same resistance profile was observed (Table 2).

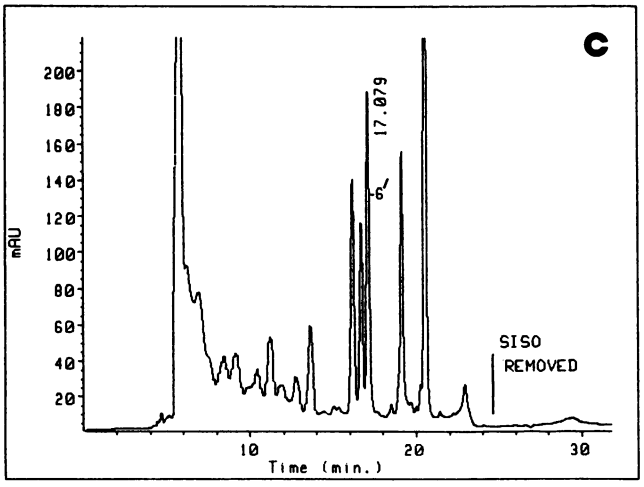
The *E. coli* transformants that contained the cloned AAC(6')-II gene on pSCH1001, pSCH1002, and pSCH1003 (Table 1) were also examined for their resistance profiles. Although the typical AAC(6')-II pattern of resistance was not observed, a low level of aminoglycoside resistance was found in *E. coli* (Table 2). An increase in the MIC was observed for gentamicin, tobramycin, 5-epi-sisomicin, and 2'-*N*-ethylnetilmicin but not for 6'-*N*-ethylnetilmicin, amikacin, or isepamicin. Increased resistance to netilmicin was not obvious. Similar results were obtained by Meyer and Wiedemann (21) when pBP30, which contains an AAC(6') gene isolated from *P. aeruginosa* 141, was transferred to *E. coli* HB101.

Several gamma-delta insertion mutations in the AAC(6')-II gene were examined. The resistance profile of one of these [C600(pSCH1002:4)] is shown in Table 2. The data indicated that the AAC(6')-II resistance profile of these mutants was completely abolished, resulting in susceptibility to all of the aminoglycosides tested.

The aminoglycoside resistance pattern of the AAC(6')-Ib gene (*aacA4*) from *S. marcescens* (33) was examined (Table 2). This AAC(6')-Ib gene and the AAC(6')-II gene from *P. aeruginosa* showed clearly different aminoglycoside resistance patterns. Since these genes were found in different







host strains, it is possible that the differences in the patterns of resistance were strain related.

To transform the AAC(6')-Ib gene into *P. aeruginosa*, the 7.6-kb *Pst*I fragment of the shuttle vector pKT212, which contains the origin of replication and the streptomycin resistance determinant (1), was ligated into the *Pst*I site of pAZ505, which contains the AAC(6')-Ib gene (33). Seven streptomycin-resistant, tobramycin-resistant *E. coli* 294 transformants were obtained and tested further. MIC analysis of all seven transformants showed an AAC(6')-I pattern in *E. coli*. One of them, 294(pSCH1012), was chosen for further study (Tables 1 and 2).

Plasmid DNA was isolated from 294(pSCH1012) and used to transform *P. aeruginosa* PAO38, selecting for tobramycin and streptomycin resistance. Nine transformants were analyzed and found to have identical AAC(6')-I MIC profiles [PAO38(pSCH1012)]. The differences in the aminoglycoside resistance profiles of the AAC(6')-Ib and the AAC(6')-II genes were not strain related (Table 2). Rather, when these two genes were cloned into a common *P. aeruginosa* host, the patterns of aminoglycoside resistance remained distinct.

HPLC analysis of the acetylated products of the AAC(6')-II enzyme. HPLC determination has been used to identify the reaction products of aminoglycoside-acetylating enzymes (15). This method first involves the enzymatic conversion of the aminoglycoside to the acetylated product. This is followed by either on- or off-line derivatization of any remaining primary amines with *ortho*-phthalaldehyde to form isoindole derivatives. The final products are then subjected to gradient chromatographic analysis on an octadecylsilane (C-18) reversed-phase support.

Figure 2A illustrates a typical chromatographic profile of a standard mixture of sisomicin and three sisomicin *N*-acetylated aminoglycosides, which were obtained after autoderivatization with *ortho*-phthalaldehyde at ambient temperature and reversed-phase chromatography (see Materials and Methods). Component designations were confirmed in each case by derivatization of standard references that resulted in single peaks at the specified retention time positions (data not shown).

HPLC analysis was performed on the reaction products of enzyme extracts prepared from *P. aeruginosa* SCH80090884 and *E. coli* strains containing pSCH1001, pSCH1002, or pSCH1003. Extracts from all of these strains acetylated sisomicin in the 6' position. Figure 2C illustrates a representative chromatographic profile that was obtained from the reaction of the AAC(6')-II enzyme from strain C600(pSCH 1003) with the substrates sisomicin and acetyl coenzyme A. Relative to the control (Fig. 2B, without acetyl coenzyme A), the sisomicin peak was absent, and a prominent peak at the expected retention time of 6'-*N*-acetylated sisomicin isoindole was observed. Chromatographic evaluations of the reaction products, obtained with extracts prepared from all of the isolates, showed that the disappearance or vast reduction of the sisomicin peak was synchronous with the emergence of the 6'-*N*-acetylated derivative.

FIG. 2. Chromatograms of *ortho*-phthalaldehyde derivatives of sisomicin and acetyltransferase reaction products. (A) Sisomicin standards acetylated in the 6', 3, and 2' positions are compared with nonacetylated sisomicin; (B) products of the enzyme from *E. coli* C600(pSCH1003) when the reaction is run without acetyl coenzyme A; (C) products of the enzyme from *E. coli* C600(pSCH1003) when acetyl coenzyme A is present in the reaction mixture. The peak at 17.079 min corresponds to the standard, 6'-acetyl sisomicin.

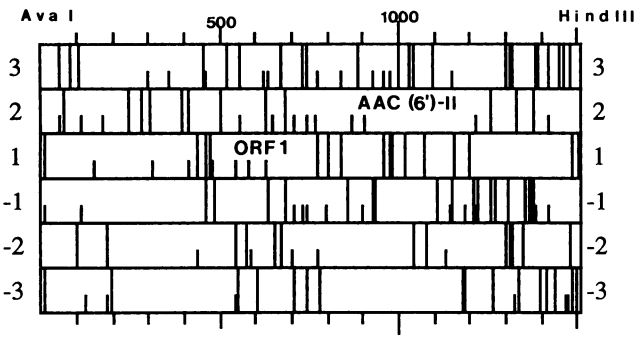


FIG. 3. Six-phase open reading frame map of the 1,507-bp *Ava*I-*Hin*dIII sequence. Reading frames 1, 2, and 3 are from left to right; reading frames −1, −2 and −3 are from right to left. Long vertical lines represent the stop codons TAA, TAG, and TGA; short vertical lines represent the methionine codon ATG.

DNA sequence analysis of the AAC(6')-II gene. Analysis of the 1,507-bp *Ava*I-*Hin*dIII sequence revealed an open reading frame between nucleotides 707 and 1258, with the coding capacity for 184 amino acids (Fig. 3 and 4). This region corresponded to the location of 9 of 10 gamma-delta insertion mutations, which destroyed the function of the AAC(6')-II gene (Fig. 1). A possible ribosome-binding site (GAG; nucleotides 694 through 696) was found 13 bp upstream of the presumed translational start site (nucleotides 707 through 709) (Fig. 4). This sequence is shorter than the average Shine-Dalgarno sequence but may be sufficient for base pairing with the 3' end of the 16S RNA (9). No promoterlike sequences were observed in this region.

Restriction analysis of the additional gamma-delta mutation placed the site of insertion nearly 200 bp upstream of the AAC(6')-II coding sequence at approximately position 516 (Fig. 4). Examination of sequences around this site revealed a second open reading frame (ORF1) from nucleotides 475 through 771 (Fig. 3 and 4). This upstream open reading frame (ORF1) overlapped with the AAC(6')-II open reading frame for 65 bp and was out of frame (Fig. 3).

The 1,507-bp DNA sequence was compared with the GenBank DNA sequence library (IntelliGenetics, Mountain View, Calif.). At least 700 nucleotides of the 5'-flanking sequence of the AAC(6')-II gene (beginning 17 bp upstream) were nearly identical (99%) to several other DNA sequences (Fig. 5). These included the 5'-flanking sequences of *aadB* [ANT(2")] (3), AAC(6')-Ia (31), *dhfrII* (30, 36), OXA2 (11), OXA1 (25), *aadA* [AAD(3")(9)] (12), and AAC(3)-I (32). These common sequences have been shown to be Tn*21* related (25). All of these sequences have the canonical sequence AAAGTT within 7 bp of the point where the DNA sequence similarity breaks down (Fig. 5B). This site has been proposed to be a hot spot for the integration of resistance genes into Tn*21*-related elements (25).

Interestingly, some of these sequences have slightly extended similarities, suggesting closer evolutionary relationships. The *dhfrII* sequence (30) and the AAC(6')-II sequence show the greatest identity, which extends 7 bp downstream of the AAAGTT sequence (Fig. 5B). Unique sequences for the ANT(2") gene begin 5 bp from this site. Likewise, AAC(6')-Ia from *C. diversus* (31), OXA1 (25), and OXA2 (7, 11) have extended sequence similarities for about seven nucleotides (Fig. 5B). Unique sequences for AAC(3)-I begin 4 bp past the AAAGTT sequence (32). The *aadA* gene has been sequenced in a Tn*21* background (12) as well as a Tn*7* background (5, 8). In this case, the sequence identity extends 11 nucleotides upstream from the initiating ATG, with the breakpoint beginning within the AAAGTT sequence. Finally, although the plasmid-borne AAC(6')-Ib gene from *S. marcescens* was found in a Tn*3* background (33), the sequence AAAGTT is found between the presumed coding region of this gene and the β-lactamase signal sequence to which it is fused (33) (Fig. 5B and 6).

The DNA sequences found immediately 3' of the AAC(6')-II gene also showed similarity to other DNA sequences. A 59-bp element was found beginning with the last codon of the AAC(6')-II gene (Fig. 4 and 5A). This element, which has been found downstream of several other resistance genes, has been suggested to contain a second insertional hotspot for recombinational events (25).

In light of the extensive sequence homologies, we examined whether Tn*21*-related sequences were associated with the AAC(6')-II gene. We found that PAO38(pSCH884) was resistant to sulfisoxazole but susceptible to streptomycin. Since the original clinical isolate SCH80090884 was resistant to sulfisoxazole and streptomycin and the transformation host PAO38 was susceptible to both drugs, it was clear that only the sulfisoxazole-resistant determinant was present on the 24-kb plasmid pSCH884. Similarly, C600(pSCH1001) (6.3-kb insert), 294(pSCH1002) (3.5-kb insert), and C600 (pSCH1003) (2.1-kb insert) were tested for resistance to sulfisoxazole. We found that only the largest plasmid, pSCH1001, was capable of conferring resistance to sulfisoxazole. This located the sulfisoxazole resistance determinant downstream of the AAC(6')-II gene (Fig. 1).

The restriction map of pSCH1001 was compared with the map of Tn*21* (13). The maps were nearly identical starting from a region in the middle of *tnpM* and extending at least 4 kb through the sequence of the sulfisoxazole resistance determinant. The only region of nonidentity was the substitution of the AAC(6')-II gene for the gene that encodes streptomycin resistance (*aadA*).

A plasmid-borne AAC(6')-Ib gene from *S. marcescens* has been cloned and sequenced (33). Both the AAC(6')-Ib and the AAC(6')-II proteins are capable of acetylating tobramycin, netilmicin, 2'-*N*-ethylnetilmicin, and 5-epi-sisomicin. In addition, the AAC(6')-Ib protein modifies amikacin, whereas AAC(6')-II modifies gentamicin. The two DNA sequences were compared and found to have 74% sequence similarity, beginning at the **TC** sequence 2 bp before the first methionine codon of the AAC(6')-II gene (Fig. 4, nucleotide 705; Fig. 6) and within the 17th codon (**ATC**) of the AAC(6')-Ib gene (33) (Fig. 6). Many of the changes were in the third codon position; as a result, 82% of the amino acid sequences are similar, allowing for conservative amino acid changes.

The 5'- and 3'-flanking sequences of these two genes are divergent. Tn*3* sequences are found upstream of the AAC(6')-Ib gene. These sequences include the promoter, the ribosome-binding site, and the first six amino acids of the β-lactamase signal peptide (33). Five nucleotides downstream of the fusion site lies the sequence AAAGTT. These sequences plus the next 20 bp show no homology to AAC(6')-II. It is possible that the original start codon for the AAC(6')-Ib gene was at the valine (GTG) codon, near where sequence similarity to the AAC(6')-II gene begins (Fig. 6).

DISCUSSION

P. aeruginosa is a clinically important pathogen comprising 32% of aminoglycoside-resistant isolates (22). The AAC(6')-II gene is one of the predominant forms of aminoglycoside resistance found associated with this organism. In

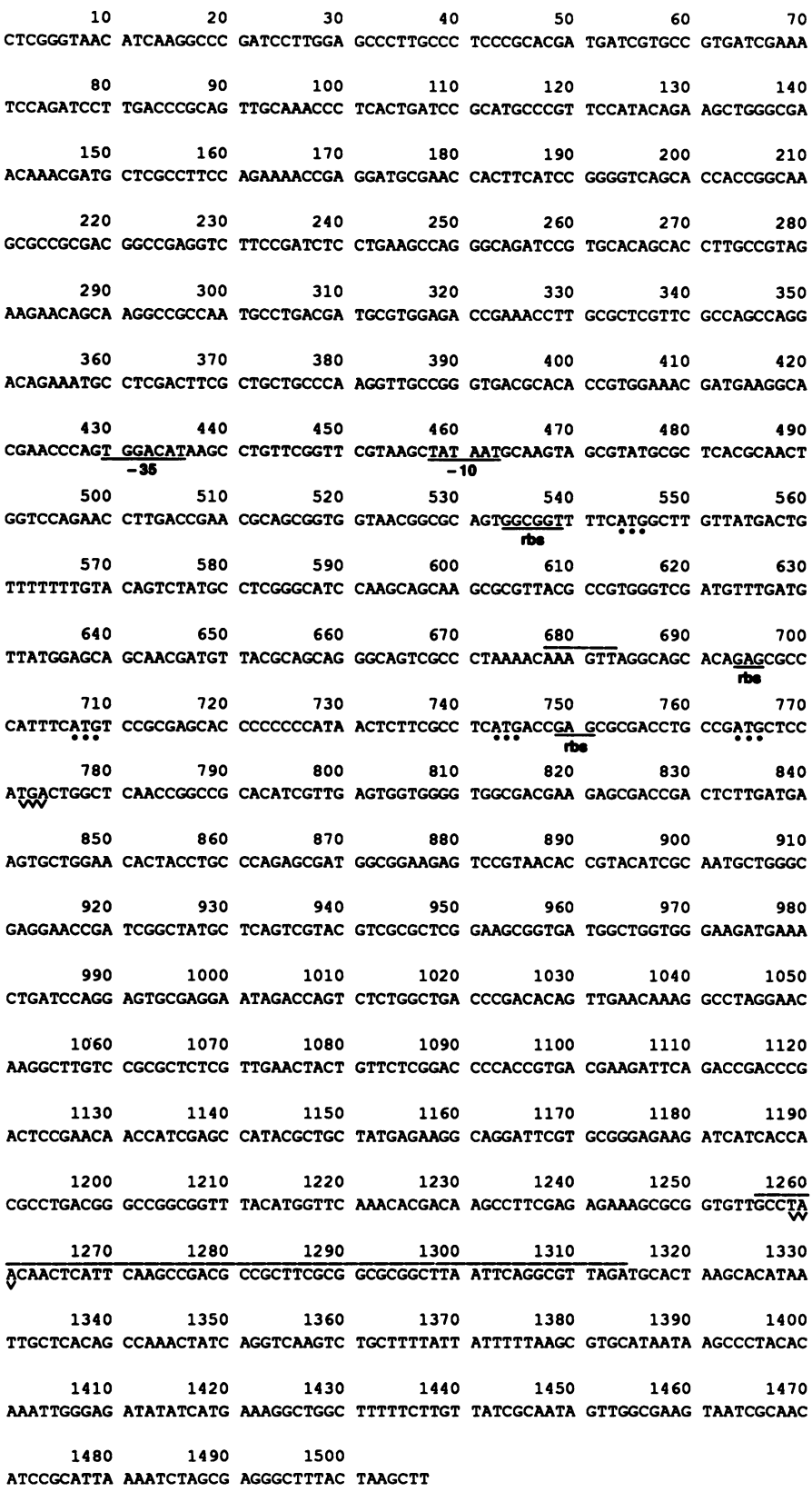


FIG. 4. Nucleotide sequence of the AAC(6′)-II resistance determinant and flanking regions. Numbering begins at the *Ava*I site and ends at the *Hin*dIII site (position 1507). Abbreviations and symbols: −10 and −35, proposed promoter sequences; rbs, possible ribosome-binding sites; ..., possible initiation codons; w, stop codons; – – – –, sequences with possible roles in recombinational events associated with Tn*21*-related elements.

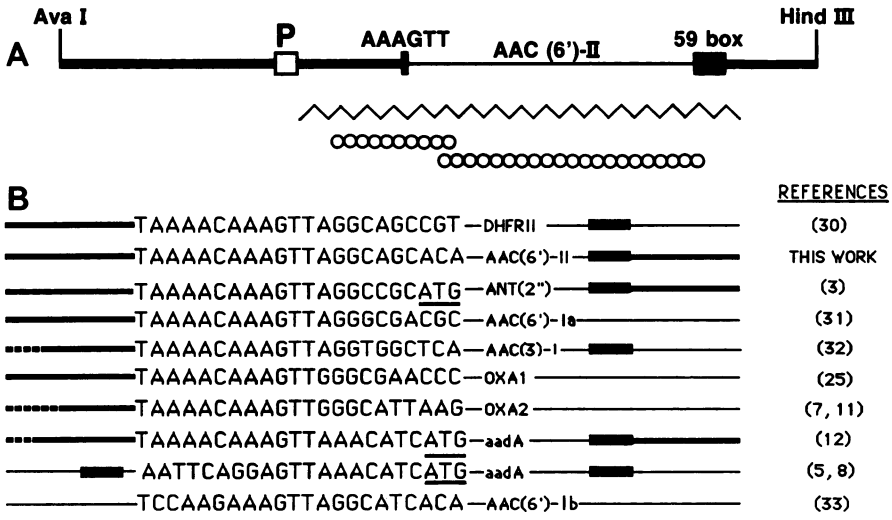


FIG. 5. Schematic structure of Tn*21*-related sequences. (A) Recombinational hot spots around the AAC(6')-II gene. Symbols: ▬, common Tn*21*-related sequences; ——, unique sequences; P, promoter; ▮, sequences suggested to play a role in recombinational events; ∿, proposed mRNA; ○○○○○, ORF1 and AAC(6')-II proteins. (B) Comparison of Tn*21* gene substitutions. Symbols: ▬, common Tn*21*-related sequences; ——, unique sequences; – – – –, not sequenced; ▮, sequences suggested to play a role in recombinational events; ATG, initiation codon.

one study of clinically isolated organisms between 1974 and 1979, the AAC(6')-II aminoglycoside resistance profile was found in 24% of resistant *Pseudomonas* isolates (22). A more recent study examined strains collected between 1978 and 1983. Of the resistant *Pseudomonas* isolates, 70% of the strains from the Far East and 29% of the strains from the United States showed this resistance profile (29). The presence of this gene leads to high level resistance to gentamicin, tobramycin, netilmicin, 5-epi-sisomicin, and 2'-*N*-ethylnetilmicin but not to amikacin or isepamicin (22). This aminoglycoside resistance profile has only been observed in *Pseudomonas* isolates (22). It is possible that this gene is on a plasmid, which only replicates in *Pseudomonas* species. However, since the expression of the AAC(6')-II resistance profile in *E. coli* is quite poor (21) (Table 2), it is possible that this gene is present in other organisms, but that these organisms would not be classified as having an AAC(6')-II resistance profile.

SCH80090884 is a strain of *P. aeruginosa* obtained from the Tokyo Clinical Research Center, Tokyo, Japan. We classified this organism by its aminoglycoside resistance profile as containing the AAC(6')-II enzyme. DNA analysis of this strain revealed the presence of a 24-kb plasmid (pSCH884) on which the AAC(6')-II gene was located.

The HPLC method for the determination of aminoglycoside enzymatic products has been previously reported (15). We have modified both the derivatization and analysis conditions and incorporated a fully automated on-line approach. This obviated the problem of precolumn instability, allowing a large number of isolates to be analyzed. HPLC analysis of sisomicin incubated with extracts of PAO38 containing plasmid pSCH884 and the AAC(6')-II subclones (pSCH1001, pSCH1002, pSCH1003) in *E. coli* indicated that this enzyme is capable of acetylating sisomicin in the 6' position.

Gamma-delta mutagenesis of the 3.5-kb AAC(6')-II subclone, pSCH1002, was performed. The location of insertion elements which inactivated the gene defined a 700-bp region representing the minimum size of the AAC(6')-II gene. Further subcloning of a 2.1-kb *Bam*HI-*Hin*dIII fragment showed that this DNA contained all of the sequences essential for the expression of this gene, including its promoter.

DNA sequence analysis of a 1,507-bp *Ava*I-*Hin*dIII fragment showed several interesting features. Of 10 gamma-delta insertion mutations, 9 were found to map to a single open reading frame (positions 707 through 1258) (Fig. 1 and 3). This open reading frame [AAC(6')-II] has the potential of encoding a protein of 184 amino acids. Upstream of the putative AAC(6')-II gene lie sequences that have also been observed abutting a number of sequences including *aadA* (12), *dhfrII* (30, 36), *aadB* (3), AAC(6')-Ia (31), OXA1 (25), OXA2 (7, 11), and AAC(3)-I (32). Included in these common upstream sequences lies an open reading frame (ORF1) (positions 544 through 771), found directly upstream of and overlapping with the AAC(6')-II gene (Fig. 3 and 4).

A DNA sequence similar to the canonical procaryotic promoter sequence (19) was observed upstream of ORF1 between positions 430 and 463 (Fig. 4). This promoter sequence was previously suggested to be utilized for expression of the *aadB* gene (3). In addition, it has been shown to be essential for expression of *dhfrII*, since linker insertion mutations located within the −35 and −10 regions disrupt the function of the *dhfrII* gene (30). A linker insertion mutation that maps between the promoter and the putative ORF1 coding sequence has also been shown to lead to the loss of function of the *dhfrII* gene (30). The function of this open reading frame is not clear; however, it has been previously shown to be fused in frame to *aadB* (3), *aadA* (12), OXA2 (7, 11), and AAC(3)-I (32). A subclone that lacks the upstream sequences, including the putative promoter and extending through nucleotide 569 (Fig. 4), does not express OXA2 (11).

In all of these cases, the first methionine codon (nucleotides 475 through 477) of a putative ORF1 polypeptide lies 12 bp away from the promoter sequence. This ATG would presumably overlap the presumptive start of transcription and is probably too close to be used. A second ATG at nucleotides 544 through 546 is more likely to be the initiating codon, and its use would result in an ORF1 polypeptide of 76 amino acids.

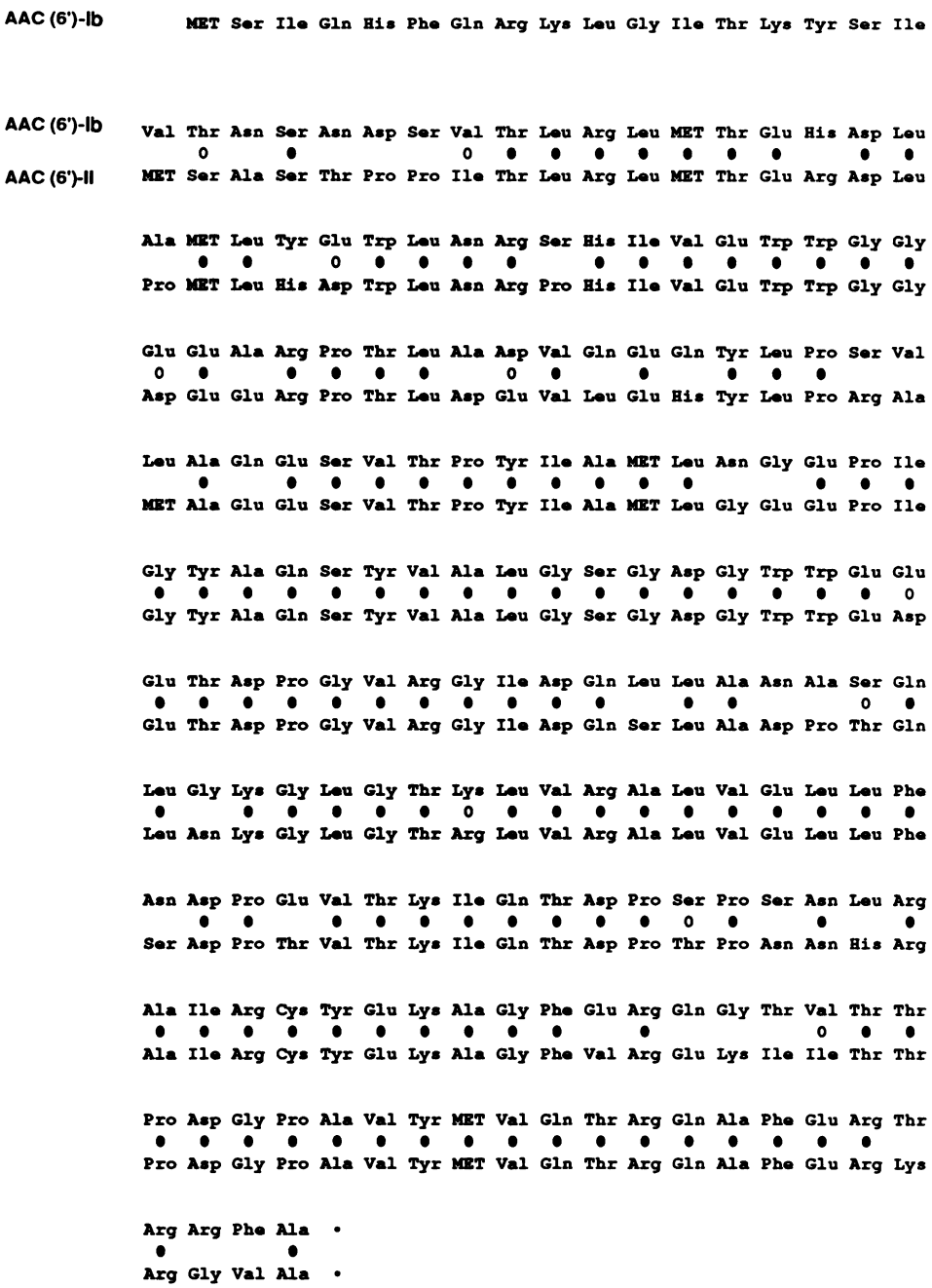


FIG. 6. Comparison of the amino acid sequences of AAC(6′)-Ib and AAC(6′)-II. Symbols: ●, identical amino acids; 0, conserved amino acid substitutions.

The AAC(6′)-II coding region contains three methionines that could potentially be used as translational starts. The resulting proteins would be 184, 172, and 165 amino acids long. Both the first and third methionines have the sequence GAG 13 bp (nucleotides 694 through 696) or 15 bp (nucleotides 749 through 751) upstream from the ATG, which could be potential ribosome-binding sites. Although these sequences are shorter than the average Shine-Dalgarno sequence, they may be sufficient for base pairing with the 3′ end of the 16S RNA (9). There is, however, no sequence resembling a procaryotic promoter sequence upstream of this coding region. It is possible that transcription initiation begins at the promoter sequence found at positions 430 through 463 and continues through the upstream ORF1 and the AAC(6′)-II gene.

Reinitiation of translation of procaryotic genes has been demonstrated to occur, provided that the stop codon for the upstream gene is within 10 bp of a good start codon and/or Shine-Dalgarno sequence for the downstream gene (9). Since ORF1 terminates within 8 bp of the third methionine codon of the AAC(6′)-II gene, and this methionine codon is preceded by a Shine-Dalgarno sequence, it is possible that the expression of the AAC(6′)-II gene is dependent upon the promoter for the upstream open reading frame. This would result in a slightly shorter AAC(6′)-II protein of 165 amino acids. This operon structure would explain two unusual

findings: (i) the polar effect of an insertion mutation located 200 bp upstream of the AAC(6')-II gene and (ii) the lack of promoterlike sequences upstream of the AAC(6')-II gene.

The AAC(6')-II 3'-flanking sequences were also shown to be very similar to other published DNA sequences (25). A 59-bp element was found, which begins with the last codon of the AAC(6')-II gene. It has been suggested that this element contains a recombinational hot spot for integration of resistance genes into Tn*21*-related elements (25). This element is within a 64-bp hairpin structure, whose stem region includes nucleotides 1255 through 1284, followed by a 3-bp loop (TTC; nucleotides 1285 through 1287) and then a stem region from nucleotides 1288 through 1318 (Fig. 4). It is possible that this large palindrome may additionally play a role in the termination of the mRNA. Hairpin-induced pausing of the RNA polymerase molecule often results in Rho-dependent termination of the mRNA (35).

The 193-bp sequence downstream of the 59-bp element is nearly identical to the sequences found downstream of the *aadA* gene (12) and the *aadB* gene (3). In the *aadA* sequence, an additional G residue is inserted 2 bp upstream of the *Hin*dIII site (12); whereas an additional A residue is found at the same location in the AAC(6')-II sequence (Fig. 4). It is likely that gene substitution is responsible for the sequence similarity found flanking these coding sequences.

The sequence of another 6'-*N*-acetyltransferase, AAC(6')-Ib, has been determined (33). This protein of 201 amino acids is 17 amino acids larger than the coding potential for the AAC(6')-II gene. However, it is likely that the N-terminal sequences are extraneous. These workers have shown that the first 6 amino acids resulted from the fusion of this gene to the N terminus of the β-lactamase gene of Tn*3*. The next 33 nucleotides include the sequence AAAGTT, which has been suggested to be an insertional hot spot found in Tn*21*-related elements (25). Sequence similarity between the AAC(6')-Ib gene and the AAC(6')-II gene begins two nucleotides upstream of the ATG (GTG) initiating codons (33) (Fig. 4, nucleotide 705; Fig. 6). Starting at this site, the two coding regions show 74% DNA similarity and 82% amino acid similarity. DNA and amino acid similarities abruptly end 555 bp downstream at the TAA termination codon (Fig. 4, nucleotide 1261; Fig. 6). The DNA sequences surrounding these genes show no similarity outside of the coding regions. It is possible that the original start codon for the AAC(6')-Ib gene was at the valine (GTG) codon, near where similarity to the AAC(6')-II gene begins (Fig. 6). The origin of the 17 N-terminal amino acids of the AAC(6')-Ib gene may be remnants of the recombinational event that inserted this gene adjacent to the Tn*3* sequences.

The high degree of sequence similarity strongly suggests that these two genes arose from a common ancestral gene. However, we now observe a difference in the spectrum of aminoglycoside resistance, even when the two genes are expressed in the same host strain. It is likely that the observed phenotypic changes have occurred during the process of dissemination of the AAC(6')-II in *P. aeruginosa* and the AAC(6')-Ib in members of the family *Enterobacteriaceae*. Several possibilities could account for the differences in resistance profiles: (i) the addition of 17 amino acids to the AAC(6')-Ib gene, resulting from the fusion to the β-lactamase signal sequence; (ii) the loss of the 19 N-terminal amino acids if translation of the AAC(6')-II protein begins at the third methionine, and (iii) one or more amino acid substitutions within the coding regions of the two genes. Experiments are in progress to distinguish which of these changes has led to the divergence in the observed aminoglycoside resistance patterns.

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