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Cloning and DNA Sequence Analysis of an *aac(3)-Vb* Gene from *Serratia marcescens*

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The AAC(3)-V resistance mechanism is characterized by high-level resistance to the aminoglycosides gentamicin, netilmicin, 2′-*N*-ethylnetilmicin, and 6′-*N*-ethylnetilmicin and moderate resistance levels to tobramycin. *Serratia marcescens* 82041944 contains an AAC(3)-V resistance mechanism as determined from aminoglycoside resistance profiles. This strain, however, does not exhibit hybridization with a probe derived from the previously cloned *aac(3)-Va* gene, (R. Allmansberger, B. Bräu, and W. Piepersberg, Mol. Gen. Genet. 198:514–520, 1985). High-pressure liquid chromatography analysis of the acetylation products of sisomicin carried out by extracts of *S. marcescens* 82041944 have demonstrated the presence of an AAC(3) enzyme. We have cloned the gene encoding this acetyltransferase and have designated it *aac(3)-Vb*. Nucleotide sequence comparisons show that the *aac(3)-Va* and *aac(3)-Vb* genes are 72% identical. The predicted AAC(3)-Vb protein is 28,782 Da. Comparisons of the deduced amino acid sequences show 75% identity and 84% similarity between the AAC(3)-Va and AAC(3)-Vb proteins. The use of a DNA fragment internal to the *aac(3)-Vb* as a hybridization probe demonstrated that the *aac(3)-Vb* gene is very rare in clinical isolates possessing an AAC(3)-V mechanism.

Resistance to the aminoglycoside group of antibiotics is often mediated by the presence of enzymes capable of acetylating, phosphorylating, or adenylylating the target aminoglycoside. The acetyltransferases are composed of three classes, defined by whether they modify the 2′, 3, or 6′ amino group of the 2-deoxystreptamine core (7, 9). The 2′-*N*-acetyltransferase, AAC(2′), appears to be limited to *Providencia* species. The gene encoding this enzyme has only recently been cloned (10). The AAC(6′) proteins are widely distributed in gram-negative bacteria. These enzymes can be classified into two groups. The AAC(6′)-I proteins are able to acetylate amikacin; however, they are unable to acetylate gentamicin. There are at least five different genes encoding these proteins (4, 13, 16, 18, 21, 22). The AAC(6′)-II proteins are capable of modifying gentamicin and are unable to modify amikacin. Presently, two genes which encode AAC(6′)-II proteins have been identified (13, 14). The third class of acetyltransferases, AAC(3), is classified into seven groups depending on the resistance profiles conferred by each protein. Representative genes, encoding most of these proteins, have been isolated (1, 3, 6, 10–13, 19, 20, 23, 24). In several instances more than one gene has been found to encode a particular class of AAC(3) proteins.

The AAC(3)-V proteins are capable of acetylating the clinically important aminoglycosides gentamicin, tobramycin, and netilmicin. In addition, they are capable of acetylating 2′-*N*-ethylnetilmicin and 6′-*N*-ethylnetilmicin. Previously, an *aac(3)-V* gene (1) has been cloned from both *Serratia marcescens* and *Klebsiella pneumoniae*. This gene, *aac(3)-Va* (also called *aacC3* and AAC(3)-III), has been shown by DNA hybridization studies to be widely distributed in gram-negative organisms (15). In addition, it was shown that some organisms expressing an AAC(3)-V resistance profile did not contain DNA which hybridized with an *aac(3)-Va* probe, indicating that an additional *aac(3)-V* gene(s) was present. To gain further information on (3)-*N*-acetyltransferases and in particular the AAC(3)-V proteins, we have characterized an *aac(3)-V* gene from a strain of *S. marcescens* which does not exhibit hybridization to the previously cloned *aac(3)-Va* gene but expresses the AAC(3)-V resistance profile. We have designated this gene *aac(3)-Vb*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* DH5α was used as a host for all DNA transformations and plasmid propagations. Plasmids pUC19 (Bethesda Research Laboratories) and pBluescript II KS− (Stratagene) were used as cloning vectors. *S. marcescens* 82041944 was obtained from the Schering-Plough Research Institute strain collection and was originally isolated at the Medical University of South Carolina. Strains used in the hybridization analysis were obtained between the years 1990 and 1991 and were isolated from the following countries: Argentina, Chile, France, Germany, Greece, Guatemala, Italy, Mexico, and Uruguay. These isolates included *Serratia* spp., *Salmonella* spp., *Pseudomonas* spp., *Providencia* spp., *Proteus vulgaris*, *Proteus penneri*, *Proteus mirabilis*, *Morganella morganii*, *Klebsiella* spp., *E. coli*, *Enterobacter* spp., *Citrobacter* spp., and *Acinetobacter* spp.

Enzymes and biochemicals. Restriction enzymes used in this study were purchased from New England Biolabs. DNA modifying enzymes were purchased from Boehringer Mannheim Biochemicals. DNA sequencing was performed by using a Sequenase kit purchased from United States Biochemical Corp. Biochemicals were purchased from Sigma Chemical Co. Aminoglycosides were obtained from the following sources: tobramycin, Eli Lilly and Co. (Indianapolis, Ind.); amikacin, Bristol Myers-Squibb Laboratories (Princeton, N.J.); and gentamicin, netilmicin, 2′-*N*-ethyl-

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TABLE 1. Aminoglycoside resistance profiles of *S. marcescens* and *E. coli* strains

Strain	MIC (μg/ml)[a]							
	GEN	TOB	AMIK	ISEP	NETIL	5-EPI	2'-NET	6'-NET
S. marcescens 82041944	64	8	4	4	32	1	128	256
E. coli DH5α	0.25	0.12	0.12	0.12	0.12	0.12	0.25	0.25
E. coli DH5α pSCH201	64	8	1	1	32	2	64	256
E. coli DH5α pSCH4203	32	8	0.25	0.25	32	1	16	64

[a] MICs were determined in microdilution plates containing 0.1 ml of Mueller-Hinton broth (plus drug). Plates were inoculated at approximately 5 × 10^{4} cells per ml and incubated at 37°C for 20 h. Abbreviations: GEN, gentamicin; TOB, tobramycin; AMIK, amikacin; ISEP, isepamicin; NET, netilmicin; 5-EPI, 5-*epi*-sisomicin; 2'-NET, 2'-*N*-ethylnetilmicin; 6'-*N*-ethylnetilmicin.

netilmicin, 6'-*N*-ethylnetilmicin, isepamicin, and 5-*epi*-sisomicin were prepared at Schering-Plough Research Institute (Bloomfield, N.J.).

Cloning of the *aac(3)-Vb* gene. Total DNA was prepared from *S. marcescens* 82041944 as described previously (8). A partial *Sau*3A digestion was performed, and fragments were ligated to pUC19 which had been digested with *Bam*HI. The ligation mixture was used to transform *E. coli* DH5α, and the resulting cell suspension was plated on Luria-Bertani agar containing ampicillin (80 μg/ml) and gentamicin (10 μg/ml). A recombinant plasmid, pSCH201, containing a 5.9-kb insert, was obtained in this manner and used for further studies. Subcloning of the *aac(3)-Vb* gene was performed by *Sau*3A partial digestion of pSCH201 and then by size fractionation in low-melting-point agarose (Sea Plaque GTG; FMC Corp). Fragments in the range of 1 to 2 kb were ligated directly in the remelted agarose (17) to pBluescript II KS− digested with *Bam*HI. This ligation mixture was used to obtain ampicillin- and gentamicin-resistant transformants. A recombinant plasmid, pSCH4203, containing a 1.6-kb insert was obtained in this manner. To construct an insertional mutation in the *aac(3)-Vb* coding region, pSCH4203 was cut at a unique *Eco*47III restriction site present approximately midway into the proposed *aac(3)-Vb* coding sequence. A kanamycin resistance cassette obtained on a 1.2-kb *Sma*I fragment from pUC4::KIXX (Pharmacia) was ligated to the *Eco*47III-linearized pSCH4203, creating pSCH4203.K.

DNA sequencing. Nested deletions of pSCH4203 were obtained by exonuclease III and S1 nuclease. DNA sequencing was performed on double-stranded plasmid templates by using a Sequenase kit (United States Biochemicals). Primers used for sequencing were T7 and T3, with internal primers used when necessary. DNA fragments were resolved by electrophoreses in 6% acrylamide gels containing 8 M urea.

HPLC analysis. Crude enzyme extracts from E. coli DH5α with and without pSCH4203 were prepared as described previously (14). Conditions for the high-pressure liquid chromatography (HPLC) analysis have been described previously (14).

DNA hybridizations. The conditions for filter preparation and DNA hybridizations have been described previously (15). The *aac(3)-Va* probe consisted of a 514-bp *Sal*I-*Cla*I fragment from pC390 (2). A 742-bp *Cla*I-*Eco*RV fragment from pSCH4203 was used as an *aac(3)-Vb* probe.

Nucleotide sequence accession number. The nucleotide sequence of the *aac(3)-Vb* gene has been assigned the GenBank accession number M97172.

RESULTS

Cloning the *aac(3)-Vb* gene. *S. marcescens* 82041944 demonstrated an AAC(3)-V resistance profile with high-level resistance to gentamicin, netilmicin, 2'-*N*-ethylnetilmicin, and 6'-*N*-ethylnetilmicin (Table 1). However, the DNA from this strain does not hybridize to the previously cloned *aac(3)-Va* gene (1) (data not shown). A recombinant plasmid, pSCH201, containing a 5.9-kb insert of *S. marcescens* DNA was isolated, which conferred an AAC(3)-V resistance profile to *E. coli* transformants (Table 1). The resistance levels of this transformant to gentamicin (64 μg/ml), netilmicin (32 μg/ml), 2'-*N*-ethylnetilmicin (64 μg/ml), and 6'-*N*-ethylnetilmicin (256 μg/ml) are nearly identical to that of *S. marcescens* 82041944. Further subcloning resulted in plasmid pSCH4203, containing a 1.6-kb insert, which also conferred an AAC(3)-V resistance profile.

HPLC analysis. To verify that pSCH4203 encoded an (3)-*N*-acetyltransferase, HPLC analysis was performed on the acetylated products of sisomicin after incubation with crude *E. coli* extracts and acetyl coenzyme A (Fig. 1). Figure 1A shows a typical chromatogram of sisomicin and three *N*-acetyl sisomicin derivatives, (2', 3, and 6'). HPLC analysis of the reaction products by using extracts prepared from *E. coli* containing pBluescript II KS− showed a prominent peak at 23.77 corresponding to the unacetylated sisomicin (Fig. 1B). There is no peak corresponding to 3-*N*-acetyl sisomicin. HPLC analysis was performed on the reaction products of sisomicin after incubation with extracts prepared from *E. coli* containing pSCH4203 (Fig. 1C). A prominent peak corresponding to the 3-*N*-acetylation product of sisomicin can be seen at position 17.2, with a corresponding loss of the sisomicin peak. This result demonstrated that 3-*N* acetylating activity is only present in *E. coli* containing pSCH4203.

DNA sequence analysis. The DNA sequence of the insert present in pSCH4203 was determined. Overall the insert was 1,572 bp in length (Fig. 2). A homology search of the cloned insert with the GenBank data base identified an open reading frame extending from nucleotide 656 to 1465, with 72% identity to the previously sequenced *aac(3)-Va* gene (aacC3) (1). This suggested the possibility that this open reading frame encoded the *aac(3)-Vb* gene. This was further supported by the insertion of a kanamycin resistance cassette into an *Eco*47III site at position 1097 within this open reading frame (Fig. 2), creating pSCH4203.K. This insertion resulted in the inability of *E. coli* containing pSCH4203.K to grow on LB plates containing gentamicin at 1 μg/ml (data not shown), whereas cells containing pSCH4203 grow well at 20 μg of gentamicin per ml. These results are consistent with the proposal that this open reading frame encodes the *aac(3)-Vb* gene as shown (Fig. 2).

Comparisons of the AAC(3)-Va and AAC(3)-Vb proteins. The deduced protein encoded by the *aac(3)-Vb* open reading frame showed 75% amino acid identity and 82% similarity to the AAC(3)-Va protein (Fig. 3A). A comparison of the

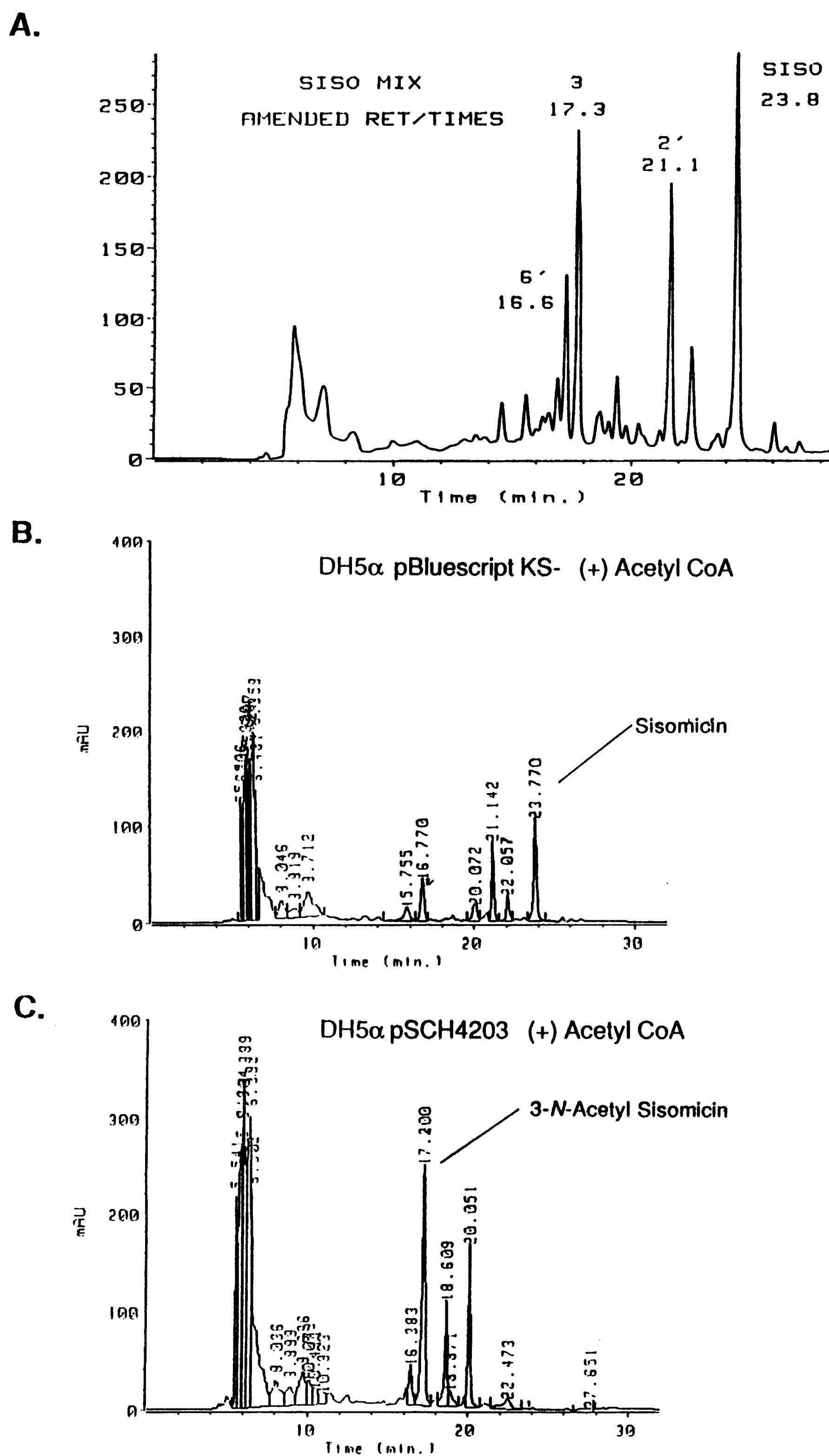

FIG. 1. HPLC chromatograms of *o*-phthalaldehyde derivatives of sisomicin and acetylated derivatives. (A) Sisomicin standards acetylated in the 6′, 3, and 2′ positions are compared with that of nonacetylated sisomicin. (B) Products of the reaction when extracts prepared from *E. coli* DH5α pBluescript II KS− were incubated with sisomicin. (C) Products of the reaction when extracts prepared from *E. coli* DH5α pSCH4203 were incubated with sisomicin. The peak at 17.2 represents 3-*N*-acetyl sisomicin.

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                                                  GGATCGAATGGCATCCGGGCGAA     22
CTGTTCCCGCGTGTCGGCTTCATCGTCACCAACCTGCCGATGGAGCCCGACTGGGTGGTGCGGTTCTATAACCAGCGCG  102
GCACCGCCGAGCAGCACATCAAGGAAGGTAAATATGCCTTCCACTGGACGCGGCTGTCATGCCGGAAGTTCCGCGACAA  181
TGAGGTGCGACTGCAACTGCATGCGCTGGCCTACAACCTGGCCATCTTCTTGCGCTGCATCGAGTTGCCCGAGACGATG  260
GCCGACTGGTCGTTGACCAGCCTGCAACTGAAGCTGATCAAGATCGGGGCACGTGTCGTCCGCCATGCCCGCGCCATCA  339
CCTTCCAGCTGGCCGAGGTGGCCGTCACCGGTCCGATGGTCCGCGCCATCCTCGCCGCCATCCGCCGATTGCGAGCGCC  418
TCCGCTATGCGCATGACCGCCATCCACGCCCAAACTGAACGAAAACGGCAGGATGGGTCTGTCCGCTGCGCTGAAAAAC  497
ACCGCCACCAGGGCACAACACGGCGGCTTCGCGGGCCGATCTGCCCTGTCTCAGCGCCTTGCGCGCCCGACGACGCCGC  576
TCGGGACGGAAAATGATTGTCCTGCGGTCGGATTCAGGCGAAACTCACGTCAGACGGCATGCCACTTGGGGAATGTATG  655

ATG AAC ACG ATC GAA TCG ATC ACG GCG GAC CTG CAC GGA CTG GGC GTC CGG CCC GGC GAC  715
Met asn thr ile glu ser ile thr ala asp leu his gly leu gly val arg pro gly asp

CTG ATC ATG GTC CAT GCA TCG CTG AAA GCC GTC GGC CCG GTC GAG GGA GGT GCG GCC TCG  775
leu ile met val his ala ser leu lys ala val gly pro val glu gly gly ala ala ser

GTG GTG TCG GCC CTT CGC GCC GCG GTC GGG TCC GCA GGG ACC CTG ATG GGT TAT GCC TCA  835
val val ser ala leu arg ala ala val gly ser ala gly thr leu met gly tyr ala ser

TGG GAC CGC TCG CCC TAT GAG GAG ACG CTG AAC GGC GCG CGG ATG GAC GAA GAA CTG CGC  895
trp asp arg ser pro tyr glu glu thr leu asn gly ala arg met asp glu glu leu arg

CGC CGG TGG CCA CCC TTC GAT CTG GCC ACA TCC GGT ACC TAT CCC GGC TTC GGC CTG CTC  955
arg arg trp pro pro phe asp leu ala thr ser gly thr tyr pro gly phe gly leu leu

AAC CGG TTT CTG CTT GAG GCG CCC GAC GCA CGG CGC AGC GCG CAT CCC GAC GCC TCC ATG  1015
asn arg phe leu leu glu ala pro asp ala arg arg ser ala his pro asp ala ser met

GTC GCG GTC GGC CCC CTT GCC GCC ACG CTG ACA GAG CCG CAC CGG CTT GGG CAG GCG CTG  1075
val ala val gly pro leu ala ala thr leu thr glu pro his arg leu gly gln ala leu
                            Eco47III
GGC GAA GGC TCG CCG CTG GAG CGC TTC GTC GGG CAT GGC GGA AAG GTC CTG CTT CTG GGA  1135
gly glu gly ser pro leu glu arg phe val gly his gly gly lys val leu leu leu gly

GCG CCG CTC GAC TCC GTC ACC GTG CTG CAT TAC GCC GAG GCC ATC GCC CCC ATC CCG AAC  1195
ala pro leu asp ser val thr val leu his tyr ala glu ala ile ala pro ile pro asn

AAA CGC CGC GTG ACC TAT GAA ATG CCG ATG CTC GGC CCG GAT GGC AGG GTC CGA TGG GAG  1255
lys arg arg val thr tyr glu met pro met leu gly pro asp gly arg val arg trp glu

CTG GCC GAG GAT TTC GAC AGC AAC GGC ATT CTC GAT TGC TTC GCG GTC GAT GGG AAG CCG  1315
leu ala glu asp phe asp ser asn gly ile leu asp cys phe ala val asp gly lys pro

GAT GCC GTC GAG ACG ATC GCC AAG GCT TAT GTC GAA CTG GGC CGG CAT CGG GAA GGC ATC  1375
asp ala val glu thr ile ala lys ala tyr val glu leu gly arg his arg glu gly ile

GTC GGT CGC GCA CCC TCC TAT CTG TTT GAA GCG CAG GAT ATC GTC TCG TTC GGC GTC ACC  1435
val gly arg ala pro ser tyr leu phe glu ala gln asp ile val ser phe gly val thr

TAT CTC GAA CAG CAT TTC GGC GCG CCC TGA TGGACGTCGTTGGCGAGAGTGAGGAGGACGCATCCGCGC  1504
tyr leu glu gln his phe gly ala pro OPA

GCAAGGCCGGGGAGACCGTTCCGGTCGAGCGGCAGCGATCAGGAAGAGGCGTTGACCCTATCGGATCC            1572
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FIG. 2. Nucleotide sequence of the cloned insert in pSCH4203. The complete nucleotide sequence of the 1,572-bp *Sau*3A insert in pSCH4203 is shown. The *aac(3)-Vb* gene begins at nucleotide 656, and the deduced amino acid sequence is shown below each codon. The location of the *Eco*47III site used to construct an insertional mutation in the *aac(3)-Vb* gene is shown at position 1097.

hydropathy profiles of the two proteins was performed by using the algorithm of Hopp and Wood (5). The AAC(3)-Vb and AAC(3)-Va proteins showed similar profiles (Fig. 3B).

Distribution of the *aac(3)-Vb* gene. In order to determine the frequency of the *aac(3)-Vb* gene in clinical isolates, we performed DNA dot blot hybridizations on clinical isolates demonstrating an AAC(3)-V mechanism as determined by aminoglycoside resistance profiles. A total of 522 organisms were probed with both the *aac(3)-Va* and *aac(3)-Vb* genes. In experiments utilizing the *aac(3)-Vb* probe, a total of 31 organisms were positive, corresponding to a frequency of 5.9%. In experiments using the *aac(3)-Va* probe, 72.2% of the organisms were probe positive.

DISCUSSION

We have cloned a new *aac(3)-V* gene, *aac(3)-Vb*, from *S. marcescens* 8802041944. The nucleotide sequence of the 1,572-bp insert has revealed a large open reading frame containing 72% nucleotide identity to the previously sequenced *aac(3)-Va* gene. An insertional mutation within this open reading frame abolished AAC(3)-V activity, providing

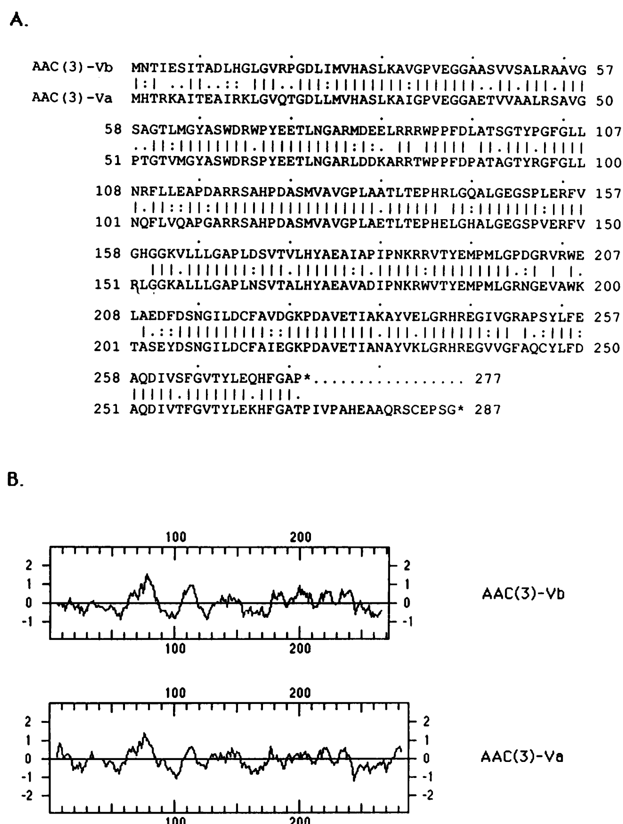

FIG. 3. Comparisons between AAC(3)-Vb and AAC(3)-Va. (A) Amino acid comparisons between the AAC(3)-Vb and AAC(3)-Va proteins. Identical amino acids between the two proteins are shown by a vertical line (|), conservative amino acid substitutions are shown by a colon (:), and amino acids with low similarity are represented by a period (.). (B) comparison of the hydropathy profiles between the AAC(3)-Vb and AAC(3)-Va proteins by using the algorithm of Hopp and Wood (5).

further support that it encodes the *aac(3)-Vb* gene. However, it is possible this insertion exerts a polar effect on a downstream gene or disrupted an open reading frame on the opposite strand.

Previous work demonstrated that among three sequenced *aac(3)-Va* genes two different upstream regions were found (1). One version, present in plasmid pWP14a, was found to contain an IS*140* element directly upstream of the *aac(3)-Va*, and the second version contained a Tn*3*-like structure upstream. We have compared the 5′ and 3′ DNA sequences flanking the *aac(3)-Vb* gene with the GenBank data base and found no significant homology to any known sequences. This indicated that *aac(3)-Vb* is present in a distinct genetic environment from that of *aac(3)-Va*. It is tempting to speculate that the *aac(3)-Va* and *aac(3)-Vb* genes are derived from a common ancestral gene and that the process of dissemination has resulted in sequence divergence as well as different genetic environments. This will, however, require the identification of this ancestral gene.

The deduced amino acid sequence of the AAC(3)-Va and AAC(3)-Vb proteins demonstrated 75% identity and 84% similarity (Fig. 3A). In addition, hydropathy profiles of the two proteins were remarkably similar (Fig. 3B). Comparisons of the amino acid sequence of AAC(3)-Vb with that of other AAC(3) proteins, including AAC(3)-Va, will provide useful information on which types of amino acids are important in maintaining the function and specificity of these proteins. For example, it is reasonable to assume that conserved amino acids between the AAC(3)-Vb and AAC(3)-Va proteins are important in determining the substrate specificities characteristic of AAC(3)-V proteins. The identification of regions within AAC(3)-Vb and AAC(3)-Va that either are conserved or differ from those of other AAC(3) proteins will also help in the determination of amino acids involved in determining the substrate specificity of various AAC(3) proteins. In addition, the identification of a conserved region(s) among the AAC(2′), AAC(6′), and AAC(3) proteins may help to identify an acetyl coenzyme A binding site.

DNA hybridization experiments utilizing an *aac(3)-Vb* probe demonstrated that *aac(3)-Vb* is very rare in clinical isolates containing an AAC(3)-V resistance profile. It comprises only 5.9% of the organisms containing this mechanism, whereas the *aac(3)-Va* gene is found in 72.2% of the isolates. The clinical isolates which contained the *aac(3)-Vb* were *Pseudomonas*, *Providencia*, *Proteus*, *Enterobacter*, and *Klebsiella* spp. and *E. coli* and were isolated from Chile, Germany, Greece, Italy, and Uruguay. The *aac(3)-Va* gene could be found in at least one isolate from each of the different species examined and was found in every country except France.

There are several reasons why this gene may be rare in clinical isolates. The DNA environment surrounding the *aac(3)-Vb* gene does not show any homology to known plasmid or transposon sequences. In addition, we have not been able to conclusively determine whether the *aac(3)-Vb* gene is plasmid or chromosomally located. If it is chromosomally located, the *aac(3)-Vb* gene may not be able to move freely between different bacterial genera. In contrast, the *aac(3)-Va* gene is both plasmid encoded and adjacent to transposon sequences, which may account for its prevalence in organisms containing an AAC(3)-V resistance profile.

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