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Primary Structure of an Aminoglycoside 6′-N-Acetyltransferase, AAC(6′)-4, Fused In Vivo with the Signal Peptide of the Tn3-Encoded β-Lactamase

GUY TRAN VAN NHIEU AND EKKEHARD COLLATZ*

Laboratoire de Microbiologie Médicale, Université Paris VI, 75270 Paris Cédex 06, France

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The gene *aacA4* encoding an aminoglycoside 6′-*N*-acetyltransferase, AAC(6′)-4, was cloned from a natural multiresistance plasmid, and its nucleotide sequence was determined. The gene was 600 base pairs (bp) long, and the AAC(6′)-4 had a calculated molecular size of 22.4 kilodaltons and an isoelectric point of 5.35. The sequence of the 17 N-terminal amino acids was determined from the purified enzyme. The AAC(6′)-4 gene was part of a resistance gene cluster, and its expression was under the control of the regulatory sequences of the β-lactamase encoded by Tn*3*. The five N-terminal amino acids were identical to those of the signal peptide of the Tn*3*-encoded β-lactamase, and the entire 5′ region of *aacA4*, as far as it was sequenced (354 bp, including the promoter and the ribosome-binding site sequences), was identical to that of the β-lactamase gene. This led us to presume an in vivo fusion between the β-lactamase and the acetyltransferase genes. The latter was followed, in a polycistronic arrangement, by an aminoglycoside 3″,9-adenylyltransferase gene, *aadA*, with an intergenic region of 68 bp. At a distance of ca. 1.3 kilobases in the 3′ direction, we found remnants of a second Tn*3*-like element specifying an active β-lactamase. At their 5′ extremities, the two incomplete copies of Tn*3*, which were in tandem orientation, were interrupted within the resolvase gene. We speculate that Tn*3*-related sequences have played a role in the process of selection and dissemination of the AAC(6′)-4 gene, which specifies resistance to amikacin and related aminoglycosides.

Bacterial resistance to aminoglycoside antibiotics occurs most frequently via drug modification, by O phosphorylation or O adenylylation or N acetylation (8). The sequence analysis of several aminoglycoside 3′-*O*-phosphotransferase genes has substantiated the notion of an evolutionary relationship between the genes found in clinical isolates (2, 15, 30, 42) and those in antibiotic-producing organisms (15, 39).

Less structural data are available for the aminoglycoside acetyltransferases, except that 3-*N*-acetyltransferases of type III and IV, both common in gram-negative bacteria, were obviously related (1) and that there was little homology between these and a 6′-*N*-acetyltransferase from a gram-positive organism (10). Nothing is known about possible structural relationships with similar acetyltransferases synthesized by aminoglycoside-producing organisms. The effect of a 6′-*N*-acetyltransferase gene on the resistance level, as well as on antibiotic production, has recently been described, and potential benefits of the introduction of similar bacterial genes into aminoglycoside-producing organisms have been alluded to previously (7).

Enzyme-mediated resistance to amikacin in gram-negative bacteria appears to depend exclusively on the synthesis of 6′-*N*-acetyltransferases of type 4, AAC(6′)-4 (17). Amikacin is a semisynthetic derivative of kanamycin A (18) and, as such, is no longer a substrate for almost all the kanamycin-modifying enzymes. Hence, its clinical importance is demonstrated.

Studying a recent episode of rapid dissemination of amikacin resistance between several enterobacterial genera, we found the resistance carried by closely related multiresistance plasmids belonging to the IncM group and specifying an AAC(6′) (41). It was the purpose of the present study to establish the primary structure of the responsible resistance determinant and to analyze adjacent nucleotide regions to obtain elements for speculation about the mechanism of the dissemination process. The latter was done in the light of what appears to be conflicting evidence that increased selective pressure may (22, 34) or may not (32, 27, 31) have an immediate effect on the selection of amikacin resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The multiple drug resistance plasmid pAZ007 which carries the gene *aacA4*, encoding the aminoglycoside acetyltransferase AAC(6′)-4, was isolated from a wild strain of *Serratia marcescens* and was previously referred to as type IA (41). Recipient strains were *Escherichia coli* BM694 for plasmid transformation and *E. coli* JM103 for transfection with M13 bacteriophage vectors. Bacteria were grown in Mueller-Hinton broth (Diagnostics Pasteur), except *E. coli* JM103, which was grown in twofold-concentrated YT broth (28).

Electrophoretic techniques. DNA fragments generated by restriction endonuclease digestion were analyzed in horizontal agarose gels in acetate buffer as described previously (23). When fragments were to be isolated, low-melting-point agarose was used. DNA fragments smaller than 600 base pairs (bp) were analyzed on vertical polyacrylamide gels containing 4.5% acrylamide and 0.15% bisacrylamide. Proteins were analyzed in sodium dodecyl sulfate-containing polyacrylamide gels by the method of Laemmli (21). Preparative electrophoresis of proteins was done under the same conditions, but the proteins were stained for only 30 min with Coomassie blue in 40% methanol. Protein from cut out

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain (genotype) or plasmid	Resistance marker(s)[a]	Reference or derivation
E. coli JM103 [F' *traD36* Δ(*lac-pro*) *thi strA supE endA sbcB15 hsdR4 proAB lacL*^{q}ΔM15]	Sm	10
E. coli BM694 (F^-)	Nal	Nal^r mutant (20) of C-1a (23)
pBR322	Pc Tc	34
pHSS6	Km	35
pAZ007	Ak Pc Sm Su Tp	Type IA (41)
pAZ500	Ak Pc Sm Tc	pBR322 linearized with *Pvu*II and ligated to a ca. 5-kb *Pvu*II fragment from pAZ007
pAZ504	Ak Pc Tc	Large *Bam*HI-*Ava*I fragment of pBR322 ligated to a ca. 2.3-kb *Bam*HI-*Ava*I fragment from pAZ500
pAZ505	Ak Pc Tc	pBR322 linearized with *Cla*I and ligated to a ca. 1.5-kb *Hpa*II fragment from pAZ504
pAZ605	Ak Pc Km Sm	pHSS6 linearized with *Sma*I and ligated to the ca. 5-kb *Pvu*II fragment from pAZ007

[a] Resistance to: Ak, amikacin and related aminoglycosides including kanamycin, via acetylation; Km, kanamycin and related aminoglycosides, but not amikacin, via phosphorylation; Nal, nalidixic acid; Pc, ampicillin; Sm, streptomycin; Tc, tetracycline; Tp, trimethoprim.

strips was electroeluted in sodium dodecyl sulfate running buffer into dialysis bags attached to Pasteur pipettes, dialyzed against water, precipitated in 80% acetone, and stored at −20°C.

Two-dimensional polyacrylamide gel electrophoresis was done by the method of O'Farrell (29).

Gene cloning and nucleotide sequencing. Restriction enzyme digestions of DNA were performed by the method of Maniatis et al. (23). Random fragments of plasmid pAZ505 (Table 1) were generated by sonicating ca. 5 μg of DNA in 200 μl of TE buffer (23) twice for 15 s each using a microtip attached to a Branson sonifier at minimal output. The DNA fragments were precipitated with ethanol and treated with the Klenow fragment of *E. coli* DNA polymerase I to generate blunt ends. Fragments of ca. 200 to 400 bp were recovered from a low-melting-point agarose gel, subcloned into M13mp8 (which was cut with *Sma*I and dephosphorylated), and identified after hybridization with the similarly isolated and radiolabeled *Eco*RI-*Hin*dIII fragment of pAZ505 which included the ca. 1.5-kilobase (kb) *Hpa*II fragment (Fig. 1). Nucleotide sequencing of the random fragments was performed by the dideoxy chain termination method of Sanger according to Messing et al. (24) and following the protocol provided by Amersham Corp. The entire sequence of the *Hpa*II fragment was determined from overlapping clones by using the shotgun DNA-sequencing program of Staden (35). The sequence was verified at least twice on either strand (Fig. 2).

Assay and purification of aminoglycoside acetyltransferase. The AAC(6') activity was assayed as previously described (13). The enzyme was purified at 0 to 4°C from 10 ml of a 100,000 × *g* supernatant (S100) obtained from BM694 (pAZ505), grown in 1 liter of medium to the mid-logarithmic phase, after sonic disruption in buffer (TNM) containing 25 mM Tris hydrochloride, 25 mM NH_4Cl, and 10 mM $MgCl_2$ at pH 7.5. The S100 was adjusted to 1 M NH_4Cl at pH 7.5 and filtered through a column (2.5 by 60 cm) of Ultrogel AcA54 (Réactifs IBF, Villeneuve-la-Garenne, France) equilibrated with TNM. The fractions containing amikacin-acetylating activity were pooled, and the acetyltransferase was purified further by chromatography on a column of immobilized neomycin which was prepared as follows.

According to the instructions of the supplier (Réactifs IBF), Ultrogel AcA34 was activated with glutaraldehyde (38), and 10 g of neomycin was incubated with ca. 150 ml of packed activated AcA34 in a rotary shaking water bath at 20°C for 18 h. A column (2 by 60 cm) was poured and washed free of unbound neomycin with three column volumes of TNM containing 2 M NH_4Cl. The column was equilibrated with TNM before use. Approximately 80 ml of TNM containing acetyltransferase was applied at a flow rate of 0.5 ml/min, and the column was washed with 10 column volumes of TNM at the same flow rate. The enzyme was eluted with TNM adjusted to 1 M NH_4Cl, precipitated with 20% (vol/vol) trichloroacetic acid, dissolved in electrophoresis buffer (21), subjected to preparative electrophoresis (see above), and stored at −20°C.

Amino-terminal amino acid analysis. The amino-terminal sequence of the purified protein was determined by automated Edman degradation in a gas-phase sequenator (model 470 A; Applied Biosystems), and the phenylthiohydantoin derivatives of the removed residues were identified by high-pressure liquid chromatography (model 120 A; Applied Biosystems). The analysis was done in the laboratory of D. Strosberg.

Enzymes and chemicals. Reagents were obtained from the following sources: restriction enzymes, DNA polymerase I, deoxy and dideoxy nucleotide triphosphates from Boehringer Mannheim Biochemicals; T4 DNA ligase from either New England BioLabs, Inc., or Boehringer Mannheim; M13 17-base primer and α-^{35}S-ATP from Amersham; nitrocellulose filters from Schleicher & Schuell, Inc., amikacin and ampicillin from Bristol Laboratories; neomycin from Diamant; and streptomycin from Spécia.

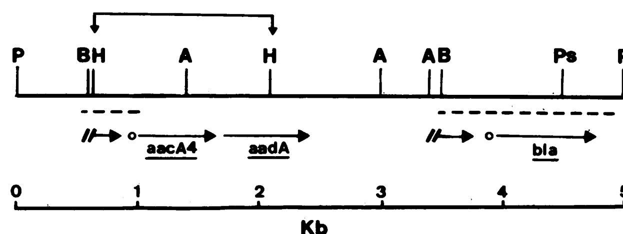

FIG. 1. Restriction map of the ca. 5-kb *Pvu*II fragment of pAZ007. The position and the direction of transcription of the genes are indicated by the horizontal arrows. The *aacA4* and *aadA* genes were positioned after subcloning of various fragments into pBR322 (see Table 1). The *bla* gene was positioned after restriction endonuclease digestion of the fragment and alignment of the *bla*-internal *Pst*I site and the upstream adjacent *Bam*HI site. Symbols: ○, promoter of the *bla* gene, designated P3 by Brosius et al. (6); //, approximate zone where the Tn*3* sequences are interrupted in the *tnpR* gene. Restriction sites are: A, *Ava*I; B, *Bam*HI; H, *Hpa*II; P, *Pvu*II; Ps, *Pst*I. The nucleotide sequence between the *Hpa*II sites indicated by vertical arrows is shown in Fig. 3.

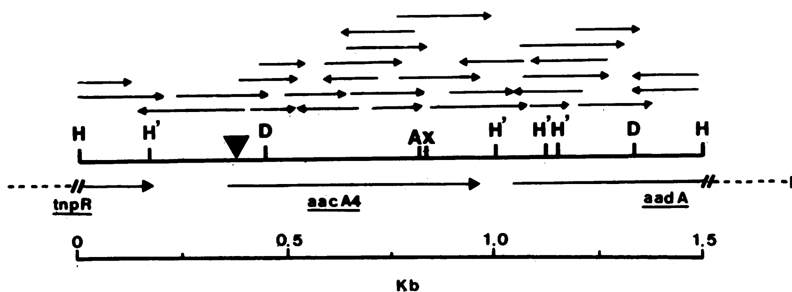

FIG. 2. Restriction map of the 1.49-kb *Hpa*II fragment of pAZ505 containing *aacA4*. The random fragments generated by sonication and the direction in which the sequence was read are indicated by the arrows at the top. ▼, Site of fusion between Tn*3* and *aacA4*. Restriction sites are: A, *Ava*I, D, *Dde*I; H, *Hpa*II; H', *Hae*III; X, *Xho*II.

RESULTS

Subcloning of the *aacA4* resistance gene. Plasmid pAZ500 was obtained after cloning of a ca. 5-kb *Pvu*II fragment from a natural wide-host-range IncM plasmid, pAZ007 (41), into pBR322 digested with the same enzyme. Plasmid pAZ500 mediated resistance to ampicillin, amikacin, and related aminoglycosides, streptomycin, spectinomycin, and tetracycline (Table 1) and conferred amikacin-acetylating and streptomycin-adenylylating activities (data not shown). These enzymes, specified by pAZ007, have previously been determined to be an AAC(6'), which fits type 4 of the nomenclature of Mitsuhashi and Kawabe (26), and an aminoglycoside adenylyltransferase, AAD(3")(8), (41). The *aacA* and *aadA* genes were closely linked on the ca. 5-kb fragment (Fig. 1) since they were contained within a 2.3-kb *Bam*HI-*Ava*I fragment obtained after partial digestion of pAZ500. This was ligated to the large *Bam*HI-*Ava*I fragment of pBR322 to yield pAZ504, and the *aacA* gene was further isolated by subcloning on a 1.5-kb *Hpa*II fragment which was ligated into the compatible *Cla*I site of pBR322 to form plasmid pAZ505 (Table 1). After sonication, this plasmid served as a source of random DNA fragments for subcloning into M13mp8 and nucleotide sequencing.

Nucleotide sequence of the *aacA4* gene. The direction and extent of the nucleotide sequences read from the recombinant phage are indicated in Fig. 2, and the sequence of 1,496 bp is shown in Fig. 3. The largest possible open reading frame, spanning 600 nucleotides, was found between the start codon ATG at positions 355 to 357 and the termination codon TAA at positions 958 to 960. The position of the start codon was confirmed after establishing the amino-terminal amino acid sequence of the purified AAC(6')-4 (see below). The deduced protein had a calculated molecular size of 22.4 kilodaltons (kDa) and an isoelectric point of 5.35. This open reading frame was followed by a second open reading frame extending beyond the nucleotide sequence determined, with the same orientation and with the possible start codon ATG at positions 1029 to 1031 or GTG at positions 1041 to 1043.

Homologies with known nucleotide sequences. The comparative analysis of the nucleotide sequence presented in Fig. 3 revealed homologies to various degrees within and adjacent to the *aacA4* gene with several published sequences. A stretch of 372 nucleotides starting at position 1 was found to be identical with that between nucleotides 3595 and 3967 of Tn*3* (14). It includes the region encoding the C-terminal moiety of the resolvase, the promoter sequence, and the ribosome-binding site sequence for the β-lactamase, as well as the sequence encoding the five N-terminal amino acids of its signal peptide (Fig. 3). These amino acids were identical with those identified at the N terminus of the amikacin acetyltransferase. This would indicate that a fusion has occurred between the two genes at the junction between nucleotides 372 and 373.

A small 63-bp sequence between nucleotides 452 and 516 was partially homologous with one of 62 bp located in the region coding for the N-terminal moiety of the AAC(3)-III described by Allmansberger et al. (1). The maximum homology was ca. 65% at the nucleotide level, and 8 of the 20 amino acids (or 9, if one considers leucine and valine equivalent) could be aligned. No similar homology was found after comparison with genes encoding an AAC(3)-IV (5), a 6'-aminoglycoside acetyltransferase-2"-aminoglycoside phosphotransferase, AAC(6')-APH(2") (10), or various chloramphenicol acetyltransferases. A large third region starting at nucleotide 632 and extending to the end of the sequenced fragment had a high degree of homology with a nucleotide sequence of the IncW plasmid Sa established by Tait et al. (37). This sequence contains the 3' end of the so-called kanamycin resistance gene, the *aadA* gene encoding a streptomycin and spectinomycin adenylyltransferase, AAD(3")(8), as well as the intergenic region. Downstream from nucleotide 663 (Fig. 3), the deduced amino acid sequences of the two acetyltransferases differed by only three amino acids, and while the intergenic region differed by the presence of only one additional nucleotide (C995 in Fig. 3), there was partial homology between the 5' terminus of the adenylyltransferase gene as far as it was sequenced (bp 1014 to 1496 in Fig. 3) and the *aad* of plasmid Sa (38). There was perfect homology, however, between the 5'-terminal sequence presented here and that of *aadA* established by Hollingshead and Vapnek (16).

Search for Tn*3* sequences on the ca. 5-kb *Pvu*II fragment. Since the *aacA4* gene was obviously fused to the region coding for the signal peptide of the β-lactamase, we searched for residues of its structural gene and further Tn*3*-derived sequences in the 3' vicinity of *aacA4*. We inserted the ca. 5-kb *Pvu*II fragment (Fig. 1) into the *Sma*I site of plasmid pHSS6. The hybrid plasmid, pAZ605, acquired the characters of resistance to amikacin, ampicillin, and streptomycin. The isolated 5-kb fragment and pBR322 were digested with the restriction endonucleases *Hae*III and *Dde*I (Fig. 4). The *Hae*III fragments A, F, and O (Fig. 4, lane a) and the *Dde*I fragments C, E, and G (Fig. 4, lane c) of pBR322 derive from the Tn*3* region including part of the *tnpR* gene, the β-lactamase gene and its regulatory sequences, and the right inverted repeat (14, 36). Fragments of identical size were obtained from the ca. 5-kb *Pvu*II fragment after digestion with the respective enzymes, i.e., *Hae*III fragments C, G, and LM (Fig. 4, lane b) and *Dde*I fragments C, D, and H (Fig. 4, lane c). The *Hae*III fragment O of 80 bp carries the promoter region of the β-lactamase and the 3' terminus of

the *tnpR* gene (6, 14). The fact that a fragment of identical mobility generated from the ca. 5-kb *Pvu*II fragment appeared as a doublet (Fig. 4, lane b) would be consistent with the existence on the 5-kb fragment of two copies of the β-lactamase promoter control region. The fact that the Tn*3*-derived *Hae*III and *Dde*I fragments mentioned above comigrated with similar fragments generated from the *Pvu*II fragment and that ampicillin resistance was mediated by plasmid pAZ605 implies the existence of an intact Tn*3*-encoded β-lactamase gene on the *Pvu*II fragment. Its position, as determined after alignment of the *bla*-internal *Pst*I and the upstream *Bam*HI sites, is indicated in Fig. 1.

If the *tnpR* genes were complete, one would expect also, at least, two copies of a 148-bp *Hae*III fragment and of a 159-bp *Dde*I fragment (14, 36) after digestion of the ca. 5-kb *Pvu*II fragment, neither of which was observed (Fig. 4). Since these fragments are located upstream of the *Bam*HI site of Tn*3* shown in Fig. 1, we conclude that both copies of the Tn*3*-like regions are interrupted within the resolvase gene.

Characterization of the 6'-*N*-aminoglycoside acetyltransferase. The AAC(6')-4 encoded by pAZ504 was identified after comparative two-dimensional polyacrylamide gel electrophoretic analysis of cytoplasmic (S100) proteins from *E. coli* BM694(pBR322) and BM694(pAZ504). The only obvious difference was the presence among the latter of a prominent, moderately acidic protein in the lower-molecular-weight zone (Fig. 5). Since the two plasmid-carrying strains were isogenic except for the presence of the *aacA4* gene on pAZ504, we concluded that the additional protein observed was the 6'-*N*-acetyltransferase.

The AAC(6')-4 was purified by a three-step procedure. After chromatography, the enzyme-containing fractions comprised, besides two or three minor proteins, one major protein which migrated after two-dimensional electrophoresis to the same position as the presumed AAC(6')-4 (data not shown). This protein was purified by preparative gel electrophoresis and had an apparent molecular size of ca. 24.5 kDa (Fig. 6). The amino-terminal amino acid sequence of the purified protein was determined by automated Edman degradation. The sequence of the 17 amino acids identified is shown in italics in Fig. 3. It corresponds to that deduced from the nucleotide sequence starting at position 358.

DISCUSSION

Cloning and sequencing of a 1,496-kb DNA fragment encoding resistance to amikacin and related aminoglycosides allowed us to establish the primary structure of the resistance determinant. The gene was identified in conjunction

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                               30                            60
CCGACAGGAAGCAAAGCTGAAAGGAATCAAATTTGGCCGCAGGCGTACCGTGGACAGGAA
 ArgGlnGluAlaLysLeuLysGlyIleLysPheGlyArgArgArgThrValAspArgAs

                               90                           120
CGTCGTGCTGACGCTTCATCAGAAGGGCACTGGTGCAACGGAAATTGCTCATCAGCTCAG
nValValLeuThrLeuHisGlnLysGlyThrGlyAlaThrGluIleAlaHisGlnLeuSe

                              150                           180
TATTGCCCGCTCCACGGTTTATAAAATTCTTGAAGACGAAAGGGCCTCGTGATACGCCTA
rIleAlaArgSerThrValTyrLysIleLeuGluAspGluArgAlaSer

                              210                           240
TTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGG

                              270                           300
GGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCG

                              330                           360
CTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGT
                                                         MetSer

            ↓                 390                           420
ATTCAACATTTCCAAAGAAAGTTAGGCATCACAAAGTACAGCATCGTGACCAACAGCAAC
IleGlnHisPheGlnArgLysLeuGlyIleThrLysTyrSerIleValThrAsnSerAsn

                              450                           480
GATTCCGTCACACTGCGCCTCATGACTGAGCATGACCTTGCGATGCTCTATGAGTGGCTA
AspSerValThrLeuArgLeuMetThrGluHisAspLeuAlaMetLeuTyrGluTrpLeu

                              510                           540
AATCGATCTCATATCGTCGAGTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGAC
AsnArgSerHisIleValGluTrpTrpGlyGlyGluGluAlaArgProThrLeuAlaAsp

                              570                           600
GTACAGGAACAGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATACATTGCA
ValGlnGluGlnTyrLeuProSerValLeuAlaGlnGluSerValThrProTyrIleAla

                              630 ↓1                        660
ATGCTGAATGGAGAGCCGATTGGGTATGCCCAGTCGTACGTTGCTCTTGGAAGCGGGGAC
MetLeuAsnGlyGluProIleGlyTyrAlaGlnSerTyrValAlaLeuGlySerGlyAsp

                              690                           720
GGATGGTGGGAAGAAGAAACCGATCCAGGAGTACGCGGAATAGACCAGTTACTGGCGAAT
GlyTrpTrpGluGluGluThrAspProGlyValArgGlyIleAspGlnLeuLeuAlaAsn
                                                   Ser
                              750                           780
GCATCACAACTGGGCAAAGGCTTGGGAACCAAGCTGGTTCGAGCTCTGGTTGAGTTGCTG
AlaSerGlnLeuGlyLysGlyLeuGlyThrLysLeuValArgAlaLeuValGluLeuLeu

                              810                           840
TTCAATGATCCCGAGGTCACCAAGATCCAAACGGACCCGTCGCCGAGCAACTTGCGAGCG
PheAsnAspProGluValThrLysIleGlnThrAspProSerProSerAsnLeuArgAla

                              870                           900
ATCCGATGCTACGAGAAAGCGGGGTTTGAGAGGCAAGGTACCGTAACCACCCCAGATGGT
IleArgCysTyrGluLysAlaGlyPheGluArgGlnGlyThrValThrThrProAspGly

                              930                           960
CCAGCCGTGTACATGGTTCAAACACGCCAGGCATTCGAGCGAACACGCAGGTTTGCCTAA
ProAlaValTyrMetValGlnThrArgGlnAlaPheGluArgThrArgArgPheAla
                                                   SerAsp

                              990                    ↓2    1020
CCCTTCCATCGAGGGGGACGTCCAAGGGCTGGCGCCTTGGCCGCCCCTCATGTCAAACGT

                             1050                          1080
TAAACATCATGAGGGAAGCGGTGATCGCCGAAGTATCGACTCAACTATCAGAGGTAGTTG
        MetArgGluAlaValIleAlaGluValSerThrGlnLeuSerGluValValG

                             1110                          1140
GCGTCATCGAGCGCCATCTCGAACCGACGTTGCTGGCCGTACATTTGTACGGCTCCGCAG
lyValIleGluArgHisLeuGluProThrLeuLeuAlaValHisLeuTyrGlySerAlaV

                             1170                          1200
TGGATGGCGGCCTGAAGCCACACAGTGATATTGATTTGCTGGTTACGGTGACCGTAAGGC
alAspGlyGlyLeuLysProHisSerAspIleAspLeuLeuValThrValThrValArgL

                             1230                          1260
TTGATGAAACAACGCGGCGAGCTTTGATCAACGACCTTTTGGAAACTTCGGCTTCCCCTG
euAspGluThrThrArgArgAlaLeuIleAsnAspLeuLeuGluThrSerAlaSerProG

                             1290                          1320
GAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCACCATTGTTGTGCACGACGACATCATTC
lyGluSerGluIleLeuArgAlaValGluValThrIleValValHisAspAspIleIleP

                             1350                          1380
CGTGGCGTTATCCAGCTAAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGACATTC
roTrpArgTyrProAlaLysArgGluLeuGlnPheGlyGluTrpGlnArgAsnAspIleL

                             1410                          1440
TTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTATCTTGCTGACAAAAG
euAlaGlyIlePheGluProAlaThrIleAspIleAspLeuAlaIleLeuLeuThrLysA

                             1470
CAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCGGCGGAGGAACTCTTTGATCCG
laArgGluHisSerValAlaLeuValGlyProAlaAlaGluGluLeuPheAspPro
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FIG. 3. Nucleotide sequence of the AAC(6')-4 resistance determinant and flanking regions. Numbering begins at the 5' end of the sequence. The deduced amino acid sequences are given below the nucleotide sequence: positions 2 to 169, C-terminal sequence of TnpR of Tn*3* (14); positions 358 to 957, AAC(6')-4; positions 1029 to 1486, putative N terminal of the AAD(3")(8) described by Hollingshead and Vapnek (16); the alternative amino acids were those identified by Tait et al. (37). The N-terminal sequences of the AAC(6')-4 determined by Edman degradation are shown in italics. Promoter-site sequences are overlined by solid lines, and the ribosome-binding site sequence is indicated by a broken line. └─┘, Zone of homology with *aacC3* (1) shown in Fig. 4; └┴┘, zone of homology with aminoglycoside 3'-phosphotransferases identified previously (37); ↓, site of fusion between Tn*3* and *aacA4*; $\downarrow^{1}$, 5' end of the zone of sequence homology described by Tait et al. (37); $\downarrow^{2}$, 5' end of a similar zone described by Hollingshead and Vapnek (16).

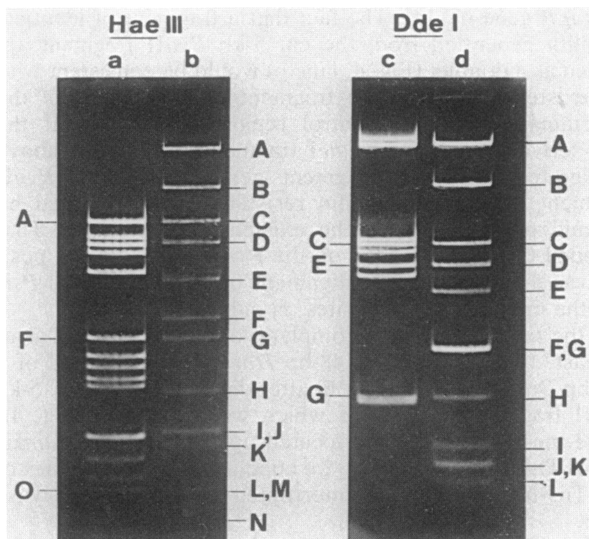

FIG. 4. Restriction endonuclease-generated fragment patterns of pBR322 and the ca. 5-kb *Pvu*II fragment of pAZ007. The enzymes used are indicated at the top. Lanes a and c, pBR322: *Hae*III fragments A, F, and O (587, 267, and 80 bp) and *Dde*I fragments C, E, and G (540, 426, and 166 bp) are designated as described (23) and derive from the Tn*3* region of pBR322 including the β-lactamase gene; lanes b and d, the isolated ca. 5-kb *Pvu*II fragment (Fig. 1).

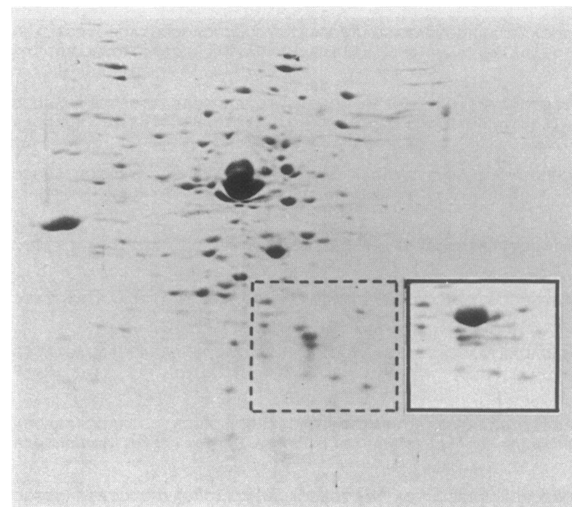

FIG. 5. Synthesis of AAC(6')-4. Cytoplasmic proteins (S100) of BM694(pBR322) (full frame) and BM694(pAZ504) (insert) were analyzed by two-dimensional polyacrylamide gel electrophoresis (29). The only obvious difference between the two sets of proteins is shown in the insert. Its area corresponds to that indicated by the broken lines in the full frame. The satellite spots on the acidic side of the prominent spot and below it were observed only with BM694(pAZ505).

with the analysis of the protein which it encodes. We could assign a 600-bp open reading frame to the *aacA4* gene, since the deduced and the determined N-terminal amino acid sequences of the AAC(6')-4 were identical and since the calculated and the estimated sizes of the protein, 22.4 and 24.5 kDa, respectively, appeared to be in fair agreement.

The search for transcriptional and translational control sequences was greatly facilitated by the observation of perfect homology of the entire 5' region (positions 1 to 372; Fig. 3) with a sequence within Tn*3* (14, 36). The facts that this sequence contains the control elements for expression of the Tn*3*-encoded β-lactamase (6), that the homology extends 18 nucleotides into the sequence encoding the signal peptide of the β-lactamase (36) and into the structural gene of the AAC(6')-4 (Fig. 3), and that the reading frame is the same in both cases led us to conclude that the AAC(6')-4 described here is the product of a gene fusion and that it is under the control of the promoter for the β-lactamase gene, which has been described as being very efficient (14). We indicated the control elements for the AAC(6')-4 accordingly in Fig. 1 and 3. We believe that the gene fusion has occurred under natural conditions, since the same sequence organization was observed on a large ca. 5-kb *Pvu*II fragment from the natural plasmid pAZ007 before subcloning of the *aacA4* gene (Fig. 1 and 4). This assumption was confirmed after analysis by restriction endonuclease digestion and DNA-DNA hybridization of plasmid DNA prepared from two clinical isolates, *S. marcescens* containing pAZ007 and one strain of *Klebsiella pneumoniae* containing a closely related plasmid (41). A *Hae*III fragment from both plasmids comigrated with the 835-bp *Hae*III fragment of pAZ505 which contains all of the AAC(6')-4 gene and the regulatory region of the β-lactamase gene (Fig. 2). All three fragments hybridized to the small *Eco*RI-*Ssp*I fragment of pBR322 containing the β-lactamase promoter region (6, 36) and also to the *Dde*I-*Hae*III fragment of pAZ505 (Fig. 2) containing most of the AAC(6') gene (data not shown). Furthermore, the acetyltransferases from *S. marcescens* carrying pAZ007 and from *E. coli* BM694 (pAZ505) were indistinguishable when probed with an anti-AAC(6')-4 antibody after two-dimensional electrophoresis and electroblotting (data not shown).

The AAC(6')-4 may be distantly related to the AAC(3)-III described by Allmansberger et al. (1), since a homology of

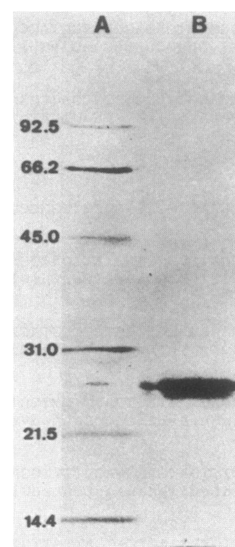

FIG. 6. Electrophoretic analysis of purified AAC(6')-4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was by the method of Laemmli (21), with an acrylamide concentration of 15%. Lane A, Molecular weight standards (Bio-Rad Laboratories) were, from top to bottom, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. Their molecular sizes in kilodaltons are indicated to the left. Lane B, AAC(6')-4. The less intense band below the major band may correspond to the satellite spots shown in Fig. 5.

ca. 65% was observed within a distinct stretch of 63 bp (Fig. 3), with some amino acid homology (see Results). There was a somewhat higher degree of homology between the amino acid sequence of the AAC(6')-4 encoded between nucleotides 765 and 814 (Fig. 3), the aminoglycoside phosphotransferase encoded by Tn*5* (2), and the phosphotransferase synthesized by *Streptomyces fradiae* (39). This observation was made by Tait et al. (37), who, studying the plasmid Sa-encoded adenylyltransferase AAD(3'')(8), cosequenced the 3' terminus of the kanamycin resistance gene but did not determine the biochemical basis of this resistance. Plasmid Sa does in fact carry an AAC(6')-4 gene (J. Davies, personal communication), but its 5'-terminal and adjacent sequences do not seem to be known. The polycistronic arrangement of the *aacA4* and *aadA* genes on pAZ505 resembles that found on plasmid Sa (37). The two intergenic regions, between nucleotides 961 and 1013 (Fig. 3), are identical with the exception of one additional nucleotide in pAZ505. The sequence downstream from position 1013, as far as sequenced, is perfectly homologous with that described by Hollingshead and Vapnek (16). The ATG codon from positions 1029 to 1031 (Fig. 3) has been suggested by these authors to be one possible initiation codon for the AAD(3'')(8). The apparent absence of promoter sequences in the intergenic region (16, 37) (Fig. 3) implies that the AAD(3'')(8) gene on pAZ007 is also under control of the β-lactamase promoter.

One AAC(6')-4 that is encoded by the plasmid pMH1 (12) has been extensively characterized. Its enzyme properties (3, 32) and its purification (32) have been reported. The enzyme described here has a similar substrate profile (26) to that reported previously (41). There is, however, no agreement with the biochemical properties reported by Radika and Northrop (32), who suggested that the pMH1-encoded AAC(6')-4 exists as a tetrameric protein of 60 to 70 kDa with a minimal subunit molecular size of 14.5 kDa.

Several AAC(6') resistance determinants have been cloned and mapped (12, 25, 40). The close spatial association, let alone fusion, with a TEM-type β-lactamase gene does not appear to be common. The fusion of the N-terminal moiety of the AAC(6')-4 to the five N-terminal amino acids of the signal peptide of the Tn*3*-encoded β-lactamase is unlikely to influence the probably intracellular localization (37) of the enzyme, since the short signal peptide segment does not contain the hydrophobic domains essential for transmembrane export (19, 43) and since it is not cleaved off the mature protein.

The incidence of amikacin resistance in several institutions has been reported to remain low over extended periods of time, although amikacin was the only aminoglycoside used (4, 27). Under a similar antibiotic usage policy, plasmid pAZ007 was isolated during the rapid dissemination of closely related multiresistance plasmids among several genera of the family *Enterobacteriaceae*, conferring resistance to amikacin (41). The dependence of *aacA4* expression, as well as that of *aadA*, on Tn*3* internal regulatory sequences suggests that the particular arrangement of genetic elements has played an ancillary role in the process of selection of the aminoglycoside resistance determinants. The existence of Tn*3*-like sequences in the upstream vicinity of the *aacC3* gene, which, incidentally, carries the *aacA4*-related stretch of 63 nucleotides (Fig. 3), has been observed on several plasmids (5), and efficient readthrough transcription from the β-lactamase promoter has been described (14). It may not be trivial that these plasmids originate from clinical strains which were isolated in the same geographical area, namely, Chile, in a period before the dissemination of the AAC(6')-4 gene (5, 34) and that AAC(3) production accounted for almost all the aminoglycoside resistance in gram-negative bacteria then observed (34).

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LITERATURE CITED

1. **Allmansberger, R., B. Bräu, and W. Piepersberg.** 1985. Genes for gentamicin-(3)-N-acetyl-transferases III and IV. II. Nucleotide sequences of three AAC(3)-III genes and evolutionary aspects. Mol. Gen. Genet. **198:**514–520.
2. **Beck, E., G. Ludwig, E. A. Auerswald, B. Reiss, and H. Schaller.** 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn*5*. Gene **19:**327–336.
3. **Benveniste, R., and J. Davies.** 1971. Enzymatic acetylation of aminoglycoside antibiotics by *Escherichia coli* carrying an R factor. Biochemistry **10:**1787–1796.
4. **Betts, R. F., W. M. Valenti, S. W. Chapman, T. Chonmaitree, G. Mowrer, P. Pincus, M. Messner, and R. Robertson.** 1984. Five-year surveillance of aminoglycoside usage in a university hospital. Ann. Intern. Med. **100:**219–222.
5. **Bräu, B., U. Pilz, and W. Piepersberg.** 1984. Genes for gentamicin-(3)-*N*-acetyltransferases III and IV. I. Nucleotide sequence of the aac(3)-IV gene and possible involvement of an IS140 element in its expression. Mol. Gen. Genet. **193:**179–187.
6. **Brosius, J., R. L. Cate, and A. P. Perlmutter.** 1982. Precise location of two promoters for the β-lactamase gene of pBR322. J. Biol. Chem. **257:**9205–9210.
7. **Crameri, R., and J. Davies.** 1986. Increased production of aminoglycosides associated with amplified antibiotic resistance genes. J. Antibiot. **39:**128–135.
8. **Davies, J., and D. I. Smith.** 1978. Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol. **32:**469–518.
9. **Deininger, P. L.** 1983. Random subcloning of sonicated DNA: application to shotgun DNA sequence analysis. Anal. Biochem. **129:**216–223.
10. **Ferretti, J. J., K. S. Gilmore, and P. Courvalin.** 1986. Nucleotide sequence analysis of the gene specifying the bifunctional 6'-aminoglycoside acetyltransferase 2''-aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and identification and cloning of gene regions specifying the two activities. J. Bacteriol. **167:**631–638.
11. **Gray, G. S., and W. M. Sitch.** 1983. Evolution of antibiotic resistance genes: the DNA sequence of a kanamycin resistance gene from *Staphylococcus aureus*. Mol. Biol. Evol. **1:**57–66.
12. **Haas, M. J., and J. Davies.** 1980. Characterization of the plasmids comprising the "R factor" R5 and their relationships to other R plasmids. Plasmid **3:**260–277.
13. **Haas, M., and J. R. Dowding.** 1975. Aminoglycoside-modifying enzymes. Methods Enzymol. **43:**611–628.
14. **Heffron, F., B. J. McCarthy, H. Ohtsubo, and E. Ohtsubo.** 1979. DNA sequence analysis of the transposon Tn*3*: three genes and three sites involved in transposition of Tn*3*. Cell **18:**1153–1163.
15. **Herbert, C. J., I. G. Giles, and M. Akhtar.** 1983. The sequence of an antibiotic resistance gene from an antibiotic-producing bacterium. Homologies with transposon genes. FEBS Lett. **160:** 67–71.

16. **Hollingshead, S., and D. Vapnek.** 1985. Nucleotide sequence analysis of a gene encoding a streptomycin/spectinomycin adenyltransferase. Plasmid **13**:17–30.
17. **Kawabe, H., T. Naito, and S. Mitsuhashi.** 1975. Acetylation of amikacin, a new semisynthetic antibiotic, by *Pseudomonas aeruginosa* carrying an R factor. Antimicrob. Agents Chemother. **7**:50–54.
18. **Kawagushi, H., T. Naito, S. Nakagawa, and K. Fujisawa.** 1972. BB-K8, a new semisynthetic aminoglycoside antibiotic. J. Antibiot. **25**:695–708.
19. **Koshland, D., R. T. Sauer, and D. Botstein.** 1982. Diverse effects of mutations in the signal sequence on the secretion of β-lactamase in Salmonella typhimurium. Cell **30**:903–914.
20. **Labigne-Roussel, A., G. Gerbaud, and P. Courvalin.** 1981. Translocation of sequences encoding antibiotic resistance from the chromosome to a receptor plasmid in *Salmonella ordonez*. Mol. Gen. Genet. **182**:390–408.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
22. **Levine, J. F., M. J. Maslow, R. E. Leibowitz, A. A. Pollock, B. A. Hanna, S. Schaefler, M. S. Simberkoff, and J. Rahal, Jr.** 1985. Amikacin-resistant gram-negative bacilli: correlation of occurrence with amikacin use. J. Infect. Dis. **151**:295–300.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Messing, J., R. Crea, and P. H. Seeburg.** 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. **9**:309–321.
25. **Meyer, J. F., B. A. Nies, and B. Wiedemann.** 1983. Amikacin resistance mediated by multiresistance transposon Tn*2424*. J. Bacteriol. **155**:755–760.
26. **Mitsuhashi, S., and H. Kawabe.** 1982. Aminoglycoside antibiotic resistance in bacteria, p. 97–122. *In* A. Whelton and H. C. Neu (ed.), The aminoglycosides. Marcel Dekker, Inc., New York.
27. **Moody, M., C. A. de Jongh, S. C. Schimpff, and G. L. Tillman.** 1982. Long-term amikacin use: effects on aminoglycoside susceptibility patterns of gram-negative bacilli. J. Am. Med. Assoc. **248**:1199–1202.
28. **Muller-Hill, B., L. Crapo, and W. Gilbert.** 1968. Mutants that make more lac repressor. Proc. Natl. Acad. Sci. USA **59**:1259–1264.
29. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
30. **Oka, A., H. Sugisaki, and M. Takanami.** 1981. Nucleotide sequence of the kanamycin resistance transposon Tn*903*. J. Mol. Biol. **147**:217–226.
31. **Price, K. E., P. A. Kresel, L. A. Farchione, S. B. Siskin, and S. A. Karpow.** 1981. Epidemiological studies of aminoglycoside resistance in the USA. J. Antimicrob. Chemother. **8**(Suppl. A):89–105.
32. **Radika, K., and D. B. Northrop.** 1984. Purification of two forms of kanamycin acetyltransferase from *Escherichia coli*. Arch. Biochem. Biophys. **233**:272–285.
33. **Seifert, S. H., M. So, and F. Heffron.** 1985. Shuttle mutagenesis: a method of introducing transposons into transformable organisms. Genet. Eng. **8**:123–133.
34. **Shimizu, K., T. Kumada, W. C. Hsieh, H. Y. Chung, Y. Chong, R. S. Hare, G. H. Miller, F. J. Sabatelli, and J. Howard.** 1985. Comparison of aminoglycoside resistance patterns in Japan, Formosa, and Korea, Chile, and the United States. Antimicrob. Agents Chemother. **28**:282–288.
35. **Staden, R.** 1979. A strategy of DNA sequencing employing computer programs. Nucleic Acids Res. **6**:2601–2610.
36. **Sutcliffe, J. G.** 1978. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43**:77–90.
37. **Tait, R. C., H. Rempel, R. L. Rodriguez, and C. I. Kado.** 1985. The aminoglycoside-resistance operon of the plasmid Psa: nucleotide sequence of the streptomycin-spectinomycin resistance gene. Gene **36**:97–104.
38. **Ternynck, T., and S. Avrameas.** 1976. Polymerisation and immobilization of proteins using ethylchloroformate and glutaraldehyde. Scand. J. Immunol. **3**(Suppl.):29–35.
39. **Thompson, C. J., and G. S. Gray.** 1983. Nucleotide sequence of a streptomycete aminoglycoside phosphotransferase gene and its relationship to phosphotransferases encoded by resistance plasmids. Proc. Natl. Acad. Sci. USA **80**:5190–5194.
40. **Tolmasky, M. E., M. Roberts, M. Woloj, and J. H. Croza.** 1986. Molecular cloning of amikacin resistance determinants from a *Klebsiella pneumoniae* plasmid. Antimicrob. Agents Chemother. **30**:315–320.
41. **Tran Van Nhieu, G., F. W. Goldstein, M. E. Pinto, J. F. Acar, and E. Collatz.** 1986. Transfer of amikacin resistance by closely related plasmids in members of the family *Enterobacteriaceae* isolated in Chile. Antimicrob. Agents Chemother. **29**:833–837.
42. **Trieu-Cuot, P., and P. Courvalin.** 1983. Nucleotide sequence of the *Streptococcus faecalis* plasmid gene encoding the 3'5''-aminoglycoside phosphotransferase type III. Gene **23**:331–341.
43. **Von Heijne, G.** 1981. On the hydrophobic nature of signal sequences. Eur. J. Biochem. **116**:419–422.