Development of a DNA Probe from the Deoxyribonucleotide Sequence of a 3-N-Aminoglycoside Acetyltransferase [AAC(3)-I] Resistance Gene

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The *aacC1* gene encoding the 3-*N*-aminoglycoside acetyltransferase [AAC(3)-I] was cloned from enteric plasmid pJR88, and its deoxyribonucleotide sequence was determined. Significant nucleotide homology was noted in the region extending from the proposed −35 sequences through the first 59 base pairs of the *aacC1* gene open reading frame (ORF) and the upstream flanking regions and ORFs of several other antibiotic resistance genes. Sequences were noted to be homologous with the 6'-*N*-aminoglycoside acetyltransferase [AAC(6')-I], 2"-*O*-aminoglycoside adenylyltransferase [AAD(2")], and 3"-*O*-aminoglycoside adenylyltransferase [AAD(3")] resistance genes; the OXA-1, OXA-2, and PSE-2 β-lactamase genes; and several dihydrofolate reductase genes. Small regions of homology were noted in the 3'-flanking regions of these resistance genes as well. A DNA probe for the *aacC1* gene was selected from the nucleotide sequence information and was tested against a series of genetically and enzymatically defined strains. The probe, which proved specific for the *aacC1* gene, was then tested against a series of 58 gentamicin-susceptible and 219 gentamicin-resistant gram-negative bacilli isolated from patients at the Seattle Veterans Administration Medical Center. Only six clinical isolates were noted to carry the *aacC1* gene. Each was resistant to gentamicin but susceptible to kanamycin, tobramycin, and amikacin. The presence of homologous regions of DNA at both the 3' and 5' ends of the *aacC1* gene reinforces the importance of choosing probes from within the ORFs of genes and of avoiding flanking sequences. When the homology with other sequences extends into the ORF, as it does with the *aacC1* gene, development of a specific probe may require determination of the nucleotide sequence.

DNA probes are useful tools for studying the epidemiology of both infectious microorganisms and antimicrobial resistance genes (52, 53). Probes directed against the sequences of the structural genes of a variety of antimicrobial resistance determinants have been used to study the dissemination of these genetic elements in hospitals and ecological communities (13, 52, 55). Since the presence of flanking sequences in probes to resistance genes can lead to false-positive results, it is preferable to select a probe from within the open reading frame (ORF) of the gene to be identified (33, 51).

Recently, Cameron et al. reported the DNA sequence of the 2"-*O*-aminoglycoside adenylyltransferase [AAD(2")] resistance gene (12) and noted that the flanking sequences of other aminoglycoside and trimethoprim resistance genes were similar to the corresponding regions upstream and downstream of the AAD(2") gene. Ouellette et al. made similar observations while investigating hot spots of recombination in transposon Tn21 (41). Such regions of homology are apparently common in transposons, such as Tn21, Tn2411, and others (31, 45). The presence of such widespread homology among resistance genes suggests that probes developed for such genes must be carefully chosen so as to avoid shared sequences.

We are interested in studying the epidemiology of the

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aminoglycoside acetylases, a diverse group of enzymes with a broad distribution in nature (5, 9, 16, 37, 39, 46). Approximately 5% of gentamicin-resistant gram-negative organisms in the United States contain the 3-*N*-aminoglycoside acetyltransferase [AAC(3)-I] gene (37). This gene has been noted in a hospital outbreak of gentamicin-resistant enteric bacilli in England (11). Although the amino-terminal end of the AAC(3)-I protein has been sequenced (26), the nucleotide sequence of the *aacC1* gene has not been reported. Herein we present the development of a DNA probe for the *aacC1* gene from its nucleotide sequence and the testing of its utility for screening a group of clinical isolates from the Seattle Veterans Administration Medical Center.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* PS4221 (Schering Corp., Bloomfield, N.J.; no. 72091801) harboring plasmid pJR88, also known as R135 and RIP135 (10, 30, 60), was obtained from R. Hare of the Schering Corp. Frozen competent cells of *E. coli* DH5α were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md. *E. coli* C600 was obtained from L. Tompkins (Stanford University, Stanford, Calif.). Both were used as recipient strains for transformation of plasmids. The cloning vectors employed, pBR322, pUC18, and pUC19, have been described previously (3). *E. coli* JM103 was the recipient strain for M13 bacteriophage vectors M13mp18 and M13mp19. The remaining study isolates are listed below (see Tables 1 to 4).

Gene cloning. Restriction endonucleases and T4 DNA ligase were purchased from New England BioLabs, Inc.,

Beverly, Mass., or Bethesda Research Laboratories and were used according to the specifications of the manufacturers. Gentamicin was provided by Schering; ampicillin and tetracycline were purchased from Sigma Chemical Co., St. Louis, Mo. Plasmid DNA was transformed into *E. coli* C600 by the method of Cohen et al. (15). Transformed *E. coli* isolates were selected for the presence of recombinant plasmids on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) or L agar containing 5 μg of gentamicin per ml and either 50 μg of ampicillin or 15 μg of tetracycline per ml. The presence or absence of the AAC(3)-I phenotype (gentamicin resistance; amikacin, kanamycin, and tobramycin susceptibility) was determined by disk diffusion (4) or microdilution MIC profiles (37). Susceptibility to sulfonamides was determined by disk diffusion with a 250-μg disk, and resistance to mercury (defined as growth in the presence of 20 μg of HgCl₂ per ml) was determined by an agar dilution method, using brain heart infusion agar.

Maxicells. Plasmid-encoded proteins were analyzed in *E. coli* maxicells (JC2926) by the method of Sancar et al. with [³⁵S]methionine (specific activity, 1,072 Ci/mmol) (43). [³⁵S]methionine and ¹⁴C-labeled molecular weight standards were purchased from Dupont, NEN Research Products, Boston, Mass.

DNA sequencing. Restriction fragments of the recombinant plasmid pFCT4374, containing the *aacC1* gene, were subcloned into phages M13mp18 and M13mp19. The DNA sequence of the insert was determined by the dideoxy-chain termination method (44) from overlapping restriction fragments, using [³⁵S]dATP (New England Nuclear Corp., Boston, Mass.) (6). Sequencing reactions were catalyzed by the Klenow fragment of *E. coli* DNA polymerase I (Pharmacia, Piscataway, N.J.) or modified T7 DNA polymerase (Sequenase; U.S. Biochemical Corp., Cleveland, Ohio) (50) and were primed with a universal M13 primer or with oligonucleotides complementary to the AAC(3)-I sequences. Oligonucleotides were synthesized on a model 380A DNA synthesizer (Applied Biosystems, Foster City, Calif.). Both

strands were sequenced throughout the ORF. To eliminate band compression on the sequencing gels, especially when sequencing regions containing inverted repeats capable of forming secondary structures, dGTP was replaced by dITP in sequencing reaction mixtures (38). In addition, the standard sequencing gel composition (35) was modified by the addition of various concentrations of formamide (6).

Computer analysis. The DNA sequence of the *aacC1* gene and the predicted gene product sequence were analyzed by using Intelligenetics software (Mountainview, Calif.) and homology search programs by Lipman and Pearson (32). The sequences were compared with the GenBank (7) and EMBL (24) nucleotide sequence data banks and the PIR (protein identification resource) protein data bank (20, 34, 40).

Probe preparation. Recombinant plasmid pFCT4392 was digested with *Ava*I, and the fragments were separated on a 1.5% agarose gel. The 845-base-pair (bp) band was excised from the gel, electroeluted into 0.1 M Tris borate buffer (pH 7.5), and purified by passage through an Elutip-d column (Schleicher & Schuell, Inc., Keene, N.H.). The DNA was then digested with *Eco*RV, and the fragments were electrophoresed through a 2.0% agarose gel. The 307-bp band was excised, purified as described above, and nick translated with ³²P, using a commercial labeling system (Bethesda Research Laboratories).

DNA hybridization studies. Duplicate nitrocellulose filters (Schleicher & Schuell) were spotted with 10 μl of organisms grown overnight in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) (ca. 10⁹ CFU/ml). Organisms were lysed as previously described (21) and were hybridized with the *aacC1* probe as described by Wahl et al. (58) at 42°C in a buffer containing 50% formamide–5× SSPE (1× SSPE is 0.15 M NaCl–0.01 M NaH₂PO₄–0.001 M EDTA)–10% dextran sulfate–10⁵ to 10⁶ cpm of ³²P-labeled probe DNA. Filters were washed three times for 5 min each in 2× SSPE–0.1% sodium dodecyl sulfate at room temperature and twice for 15 min each in 0.1× SSPE–0.1% sodium dodecyl sulfate at 50°C. Filters were air dried and exposed to X-ray
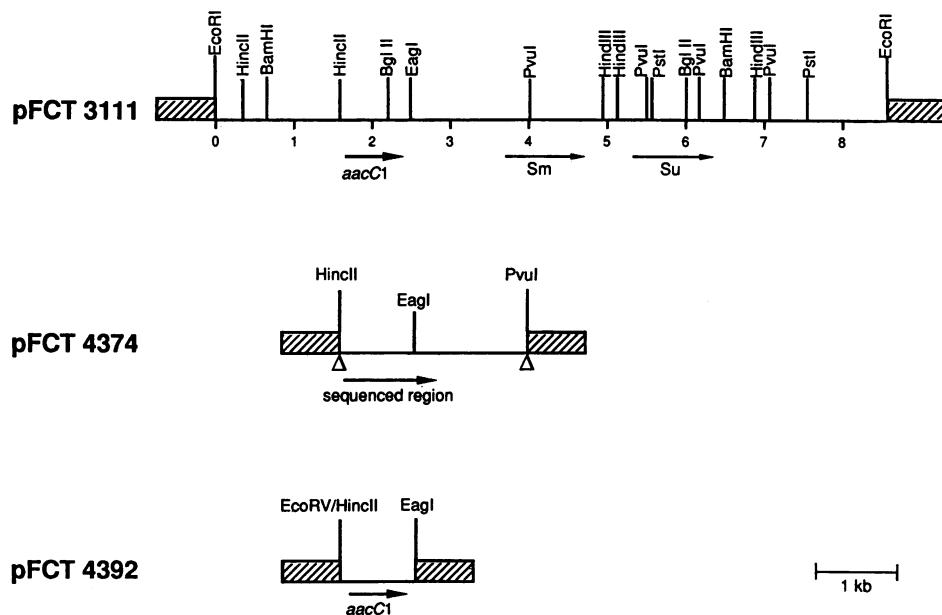

FIG. 1. Restriction endonuclease maps of cloned DNA from pJR88. pJR88 DNA (———), portions of pBR322 DNA (▨), locations of the antimicrobial resistance determinants (→), and deletions of DNA segments (Δ) are indicated. Sm, Streptomycin; Su, sulfonamide; kb, kilobase.

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GTCGACATAAGCCTGTTCGGTTCGTAAACTGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCCAGAAC    72
  -35                     -10
CTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGTACA   144

GTCTATGCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAA   216
                                                              RBS  ----
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        M   L   R   S   S   N   D   V   T   Q   Q   G   A   X   P   K   T
        :   :   :   :   :   :   :   :   :   :   :   :               :   :   :
        M   L   R   S   S   N   D   V   T   Q   Q   G       S   R   P   K   T
     CG ATG TTA CGC AGC AGC AAC GAT GTT ACG CAG CAG GGC AGT CGC CCT AAA ACA   269
     -------------------->----------------------->
        K   L   G   G
        :   :   :   :
        K   L   G   G   S   S   M   G   I   I   R   T   C   R   L   G   P   D
     AAG TTA GGT GGC TCA AGT ATG GGC ATC ATT CGC ACA TGT AGG CTC GGC CCT GAC   323
            *end 5'homology
        Q   V   K   S   M   R   A   A   L   D   L   F   G   R   E   F   G   D
     CAA GTC AAA TCC ATG AGG GCT GCT CTT GAT CTT TTC GGT CGT GAG TTC GGA GAC   377

        V   A   T   Y   S   Q   H   Q   P   D   S   D   Y   L   G   N   L   L
     GTA GCC ACC TAC TCC CAA CAT CAG CCG GAC TCC GAT TAC CTC GGG AAC TTG CTC   431
                                                         AvaI
        R   S   K   T   F   I   A   L   A   A   F   D   Q   E   A   V   V   G
     CGT AGT AAG ACA TTC ATC GCG CTT GCT GCC TTC GAC CAA GAA GCG GTT GTT GGC   485

        A   L   A   A   Y   V   L   P   K   F   E   Q   A   R   S   E   I   Y
     GCT CTC GCG GCT TAC GTT CTG CCA AAG TTT GAG CAG GCG CGT AGT GAG ATC TAT   539

        I   Y   D   L   A   V   S   G   E   H   R   R   Q   G   I   A   T   A
     ATC TAT GAT CTC GCA GTC TCC GGC GAG CAC CGG AGG CAA GGC ATT GCC ACC GCG   593

        L   I   N   L   L   K   H   E   A   N   A   L   G   A   Y   V   I   Y
     CTC ATC AAT CTC CTC AAG CAT GAG GCC AAC GCG CTT GGT GCT TAT GTG ATC TAC   647

        V   Q   A   D   Y   G   D   D   P   A   V   A   L   Y   T   K   L   G
     GTG CAA GCA GAT TAC GGT GAC GAT CCC GCA GTG GCT CTC TAT ACA AAG TTG GGC   701

        I   R   E   E   V   M   H   F   D   I   D   P   S   T   A   T   ***
     ATA CGG GAA GAA GTG ATG CAC TTT GAT ATC GAC CCA AGT ACC GCC ACC TAA CAAT   756
                                     EcoRV
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TCGTTCAAGCC GAGATCGCTTCGCGGCCGCGGAGTTGTTCGGAAAAATTGTCACAACGCCGCGGCCGCAAAG   828
                          EagI
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CGCTCCGGCTTAACTCAGGCGTTGGGCAACAAGAAAACCGATATGAACGTACGCACTTGCACTGAATCTGAC   900

GTCGCCTCTATCGCAGTCGTATTTACTGAGTCTATTCATGTACTTGGAGCGTCTCACTATGACGCTTCGCAA   972

AGGAATGCGTGGGCACCGCGTCCCGCAGATATAGAGGCTTGGTCAGCTCGCTTATCTGGCCTACAGACTCTT  1044

CTAGCAATTGAGGGAGATGCGGTTATCGGGTTCATCTC                                    1082
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FIG. 2. DNA sequence of the *Hinc*II-*Pvu*I fragment of pFCT4374. Proposed −35, −10, and ribosomal binding sites (RBS) are underlined. Direct repeats (- - - -→) and insertions of DNA downstream of ORF (——) are indicated. The top row of letters represents single-letter amino acid codes for the polypeptide determined by Hsiang et al. (26). The bottom row of letters represents amino acids predicted by DNA sequence (X, unknown amino acid). The end of homology with other resistance determinants (*) is indicated. The *aacC1* DNA probe is the fragment bounded by the *Ava*I and *Eco*RV sites.

film (X-Omat AR; Eastman Kodak Co., Rochester, N.Y.) with intensifying screens at −70°C for 24 to 48 h.

RESULTS

Cloning and nucleotide sequence determination of the AAC(3)-I gene. To facilitate the development of a specific DNA probe, the *aacC1* gene was cloned from the enteric plasmid pJR88 by inserting an 8.5-kilobase *Eco*RI fragment from pJR88 into the *Eco*RI site of pBR322. The resulting 12.8-kilobase plasmid was named pFCT3111 (Fig. 1). The restriction endonuclease map of the insert is similar to that of Tn21, described by Kratz et al. (31). Indeed, this plasmid confers both sulfonamide and streptomycin resistance to its host strain, as is common for Tn21-like elements (31, 45). A mercury resistance determinant does not appear to be present on pFCT3111, but pJR88 does contain a mercury resistance gene, adding support to the presence of a Tn21-like element on pJR88. Two consecutive deletions of pFCT3111 generated by *Pvu*I and *Hinc*II digestion produced

plasmid pFCT4374 (Fig. 1), which conferred the gentamicin resistance (Gm^r) phenotype to recipient *E. coli* cells after transformation. A 780-bp *Hinc*II-*Eag*I fragment from pFCT4374 was subcloned into the 3.6-kilobase *Eag*I-*Eco*RV fragment of pBR322, producing a 4.4-kilobase plasmid, designated pFCT4392 (Fig. 1), that produced the Gm^r phenotype when transformed into *E. coli* recipients.

The nucleotide sequence of 1,082 bp of the *Hinc*II-*Pvu*I fragment from pFCT4374 which includes the *aacC1* gene and flanking sequences was determined (Fig. 2). The overall base composition of the sequenced region is 22.7% A, 26.5% C, 26.7% G, and 24.1% T. Nucleotides 2 to 7 and 25 to 30 correspond to the −35 and −10 sequences of the *aadB* gene described by Cameron et al. (12) while the sequence GGAG at nucleotides 207 to 210 probably represents the ribosomal binding site. Comparison of the predicted amino acid sequence with the previously determined amino-terminal sequence of the *aacC1* protein encoded by RIP135 (26) indicates that the ATG at nucleotides 219 to 221 is the initiation

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aacC1  (pFCT4374)  GTCGACATAAGCCT-GTTCGGTTCGTAAACTGTAATGCAAGTAGCGTA--TGCGCTCACGCAACTGGTCCAGAACCTTGACCGAA
aacA1  (pFCT1163)  ..G........................G.........................................................
aadB   (pDGO100)   ..T.................................................................................
OXA-1  (RGN238)    ..G........................G.........................................................
dhfrII (pMT100)    ..T.................................................................................
dhfrII (R388)      ..T.................................................................................
aadA   (R538-1)    ..G...........C............G..................AC.....................................
dhfrII (R751)      ..T.............................................................................C...
OXA-2  (R46)       ..G...........C.....................................................................
dhfrV  (pLMO20)    ..G........................G.........................................................
PSE-2  (pMON234)

-CGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGAC-TGTTTTTTTG---TACAGTCTATGCCTCGGGCATCCAAGC-AGC-AAGCGCGT
.........................................................GGG........................................
....................................................................................................
.........................................................GGG........................................
....................................................................................................
...................................................-..................T...T........................
..................................................A.........GGG......................................
A....A..............................................................................................
-....G...........................................................C..................................
....................................................................................................
....................................................................................................

TACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGTGGC
.......................................................----------------............................GC.A
.......................................................----------------.............................CC..
.......................................................----------------.............................C.AA
.......................................................----------------.............................CA..
...........................A...........................----------------...T.........................CA..
.......................................................----------------...........................AACAT.
.......................................................----------------.............................CTT.
.......................................................----------------..........................G..CATT
.......................................................----------------............................ACCC.G
.......................................................----------------............................CCAC.
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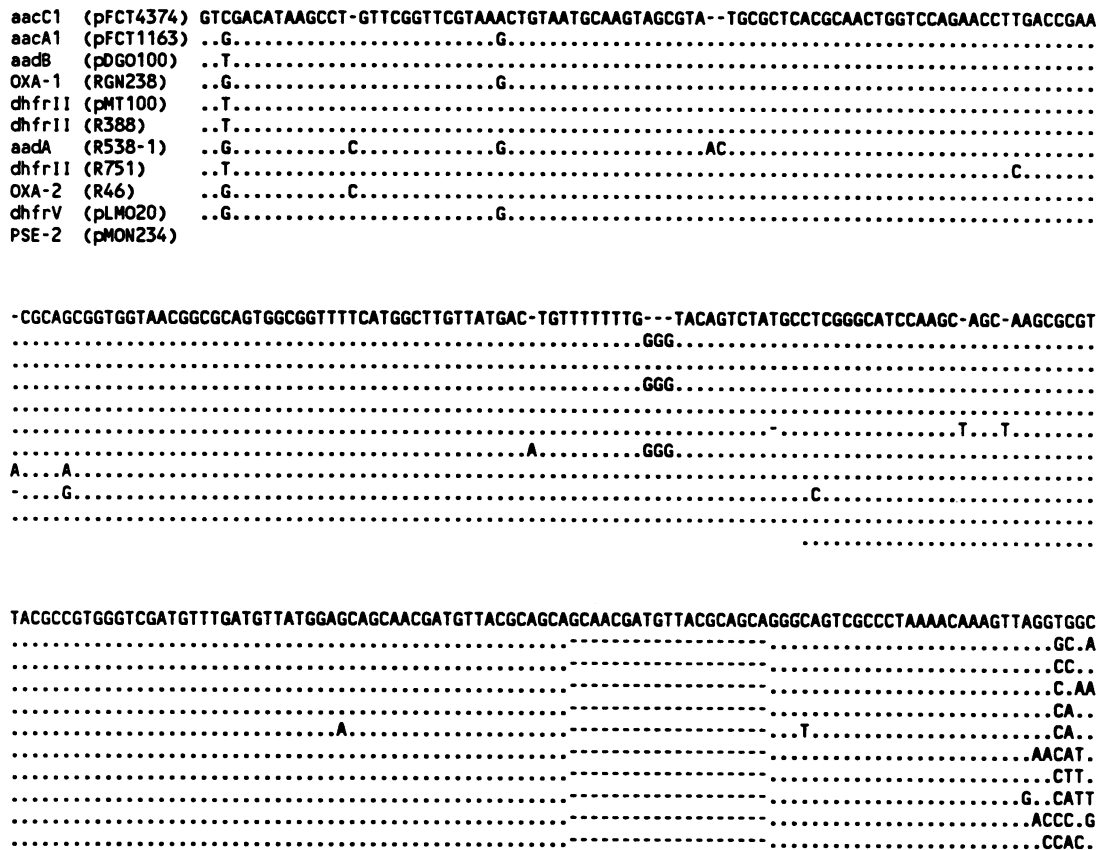
FIG. 3. DNA sequences of the 5' ends of the genes indicated. The sequence data from the PSE-2 gene start at the *Ava*I site. Dots indicate identity with *aacC1*, whereas dashed (raised) lines represent the absence of a nucleotide at a particular sequence.

codon (Fig. 2). Only two differences were noted between the amino acid sequence predicted from the DNA sequence and that which was previously determined by biochemical methods. First, the DNA sequence predicts a serine residue in place of an alanine at nucleotides 255 to 257. Second, an arginine residue is predicted at nucleotides 258 to 260 which was previously reported as an unidentifiable amino acid (Fig. 2, top row of amino acids). A translation termination codon (TAA) is located at nucleotides 750 to 752. This results in an ORF of 549 nucleotides and predicts a 177-amino-acid protein with a molecular weight of 19,388. Deletion of the 54-bp *Eco*RV-*Eag*I fragment (bp 729 to 780), which encompasses the TAA termination codon, inactivates the *aacC1* gene. This novel plasmid, pFCT4400, no longer confers the Gm^r phenotype to *E. coli* transformants containing the altered plasmid.

A search of the PIR (release 14.0) data bank failed to reveal any significant matches with other protein sequences. Specific searches of the protein sequences encoded by drug resistance genes *dhfr*II (dihydrofolate reductase type II) from R388 (62), *aadA* from R538-1 (25), OXA-2 from R46 (23), *dhfr*II from R751 (18), *aadB* from pDGO100 (12), *aacA4* from pAZ007 (57), *aacC4* from pWP7b (8), *aacC3* from three plasmids (1), *aadA* from pCN1 (14), OXA-1 from RGN238 (41), and *aacA1* from pFCT1163 (54) were unrevealing.

Expression of plasmid-encoded proteins in maxicells. To confirm that the protein produced by the ORF of the cloned *aacC1* gene was similar to that produced in vivo and to confirm the direction of transcription of the *aacC1* gene, the proteins encoded by plasmids pFCT3111, pFCT4374, pFCT4392, and pFCT4400 were examined in *E. coli* maxi-

cells. Autoradiographs of 15 to 18% sodium dodecyl sulfate-polyacrylamide gels showed a similar 19-kilodalton protein in maxicell preparations from the three Gm^r clones pFCT3111, pFCT4374, and pFCT4392 (data not shown). This 19-kilodalton protein was the only non-pBR322-derived protein in these preparations and correlates well with the 19,388-dalton polypeptide predicted by using the first ATG codon in the 549-nucleotide ORF. Preparations from gentamicin-susceptible maxicells containing pFCT4400 demonstrated a protein similar in size to that produced by the three Gm^r strains.

Nucleotide sequence homologies. A comparison of the first 277 bp of the *aacC1* sequence from the *Hinc*II site at 0 through the first 59 bp of the ORF (bp 277) with the 5'-flanking regions of a number of plasmid-borne genes that mediate antimicrobial resistance revealed extensive sequence homologies (Fig. 3). These genes include the *aacA1* 6'-*N*-aminoglycoside acetyltransferase [AAC(6')-I] gene on pFCT1163, the *aadB* gene on plasmid pDGO100, the OXA-1 gene on RGN238, the *dhfr*II gene on pMT100 (49), the *dhfr*II gene from R388, the *dhfr*II gene from R751, the *aadA* gene from R538-1, the OXA-2 gene on R46, the *dhfr*V gene on pLMO20 (48), and a portion of the PSE-2 gene from pMON234 (27). A comparison of the sequence data suggests that the *aadB* gene and the dihydrofolate reductase genes from pMT100, R388, and R751, as well as the OXA-2 gene and *dhfr*V gene, share the −35 and −10 sites with *aacC1*, while the AAC(6')-I gene, the OXA-1 gene on RGN238, and the *aadA* from R538-1 utilize the −35 and −10 sequences downstream at nucleotides 124 to 130 and 148 to 153 (Fig. 3), where the insertion of the nucleotides GGG creates the
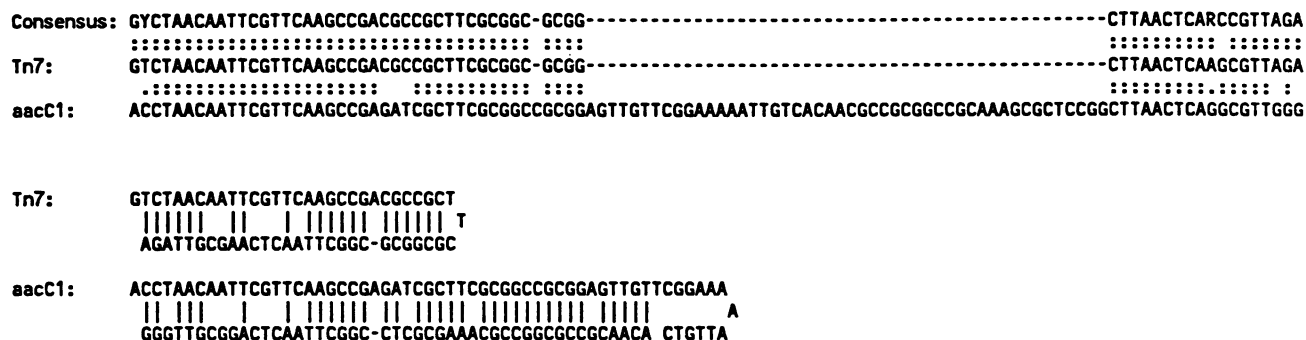
```
Consensus: GYCTAACAATTCGTTCAAGCCGACGCCGCTTCGCGGC-GCGG--------------------------------------------CTTAACTCARCCGTTAGA
           ::::::::::::::::::::::::::::::::::::::::: ::::                                                  ::::::::::: :::::::
Tn7:       GTCTAACAATTCGTTCAAGCCGACGCCGCTTCGCGGC-GCGG--------------------------------------------CTTAACTCAAGCGTTAGA
           .::::::::::::::::::::::: :::::::::::: ::::                                                  ::::::::::.::::: :
aacC1:     ACCTAACAATTCGTTCAAGCCGAGATCGCTTCGCGGCCGCGGAGTTGTTCGGAAAAATTGTCACAACGCCGCGGCCGCAAAGCGCTCCGGCTTAACTCAGGCGTTGGG
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Tn7:       GTCTAACAATTCGTTCAAGCCGACGCCGCT
           |||||| ||  | ||||||| |||||| T
           AGATTGCGAACTCAATTCGGC-GCGGCGC
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aacC1:     ACCTAACAATTCGTTCAAGCCGAGATCGCTTCGCGGCCGCGGAGTTGTTCGGAAA
           || |||  |    | ||||||| || ||||| |||||||||| |||||       A
           GGGTTGCGGACTCAATTCGGC-CTCGCGAAACGCCGGCGCCGCAACA CTGTTA
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FIG. 4. (Top) Comparison of the 3' hairpin region of the consensus sequence proposed by Cameron et al. (12), the sequence of the hairpin structure 3' to the *dhfr*II gene on Tn7 (19), and the *aacC1* sequence. The 48-bp insertion in the *aacC1* gene (---) is shown. (Bottom) Potential hairpin formation by Tn7 and the *aacC1* gene. Y, Pyrimidine; R, purine.

optimal 17-bp spacing between the −35 sequence TTGTTA and the −10 sequence TACAGT. Nucleotides 213 to 250 of the *aacC1* gene are composed of a pair of 19-base sequences which are direct repeats of one another. This direct repeat, which has not been observed in the sequence of the 5'-flanking regions of other drug resistance genes, results in a 19-base insertion with respect to the other genes (Fig. 3). Neither leader sequences nor signal peptides appear to be encoded by these shared regions.

In addition to the sequence similarity observed in the 5'-flanking region of the ORF, similarities in the region 3' to the *aacC1* gene were also noted with the above genes. Cameron et al. (12) proposed a consensus sequence of the 3'-flanking sequences that was 54 to 59 bp in length. This consensus sequence and the Tn7 repeat of Fling et al. (19) are compared with the corresponding region of *aacC1* in Fig. 4. Again, the *aacC1* sequence displays an insertion relative to the other sequences. This insertion, 48 bp in length, encompasses nucleotides 789 to 836 (Fig. 2). Even with this insert, the 3' flank of the *aacC1* gene is still capable of forming a hairpin structure, as are the other sequences. However, in this case, the hairpin encompasses 107 nucleotides rather than 54 or 59 (Fig. 3) and is potentially more stable. The potential structure is formed by a pair of sequences, each 47 nucleotides in length, which together form an imperfect inverted repeat.

Finally, the 5' recombinational hot spot (AAAGTT) described by Ouellette et al. (41) as the site of integration of resistance genes in Tn*21*-like elements is located in the *aacC1* sequence at nucleotides 269 to 274, while the 3' hot spot (GGCGTT) is at nucleotides 846 to 851.

Probe development. A restriction endonuclease map generated from the sequence of the *aacC1* gene revealed a 307-bp *Ava*I-*Eco*RV fragment within the ORF that did not overlap any of the shared sequences noted above (Fig. 2). This fragment was purified, labeled, and hybridized to DNA obtained from a series of genetically defined control strains harboring a variety of aminoglycoside resistance genes (Table 1). The probe hybridized only with the DNA of the strain demonstrating AAC(3)-I activity (PS886). Second, the DNAs from a series of reference strains demonstrating 3'-acetylating activity by phosphocellulose paper-binding assay (22) or MIC profile (37) were examined with the probe (Table 2). Homology was noted with four strains of *Pseudomonas aeruginosa*. One was previously classified as an AAC(3)-I-producing strain, another as an unclassified AAC(3) producer, the third as an AAC(3)-III producer, and the last as an AAC(3)-Ia producer. The remaining isolates in this group tested negative with the probe. A third group of 25 isolates obtained from the Seattle Veterans Administration Medical Center, which had been characterized by the MIC profile method, was examined (Table 3). Only two strains,

TABLE 1. Control strains for DNA probe studies

Strain	Organism (plasmid)	Aminoglycoside-modifying enzymes[a]	Source[b] (reference)
1009	*Providencia stuartii*	AAC(2') + AAD(2")	Bristol
886	*Escherichia coli*(pJR88)	AAC(3)-I	Schering (30, 60)
892	*Serratia marcescens*	AAC(3)-II	Schering
743	*Escherichia coli*(pMB8::Tn*1700*)	AAC(3)-III + APH(3')-I	C. Rubens (42)
1685	*Klebsiella pneumoniae*	AAC(3)-IV + APH(3')-I + APH(3')-II	Bristol
2225	*Escherichia coli*(pBWH100)	AAC(6')-I	K. Mayer (36)
823	*Serratia marcescens*	AAC(6')-II	Bristol
873	*S. marcescens*	AAC(6')-III	Schering
1	*S. marcescens*(pLST1000)	AAD(2") + AAC (6')-I	SVAMC (55)
1649	*Escherichia coli*(pIP71a)	AAD(3")	PRC (17)
1752	*Staphylococcus aureus*	AAD(4')	Schering
1688	*Pseudomonas aeruginosa*	AAD(4')-II	Bristol
1323	*Escherichia coli*(pGH54)	APH(3')-I	N. Grindley (59)
1750	*E. coli*(pSAY16)	APH(3')-II	SVAMC (61)
2727	*Staphylococcus aureus*	APH(3')-IV	Schering
736	*Escherichia coli*(pBR322)	None	L. Tompkins (3)

[a] As identified by phosphocellulose paper-binding assays (22) performed by Bristol Laboratories, Syracuse, N.Y., or MIC typing (37) performed by Schering-Plough, Bloomfield, N.J. AAC, 2'-*N* (or 4'-*N*)-Aminoglycoside acetyltransferase; APH, 3'-*O*-aminoglycoside phosphotransferase.

[b] C. Rubens, Children's Hospital, Seattle, Wash.; K. Mayer, Memorial Hospital, Providence, R.I.; SVAMC, Seattle Veterans Administration Medical Center; PRC, Plasmid Reference Center; N. Grindley, Yale University, New Haven, Conn.; L. Tompkins, Stanford University, Stanford, Calif.

TABLE 2. Hybridization of *aacC1* probe with DNA from enzymatically defined reference strains with AAC(3) activity

Strain	Organism	Enzyme	*aacC1* probe hybridization[a]	Source[b] (reference)
896	*Pseudomonas aeruginosa*	AAC(3)-I	+	Schering (9)
1683	*P. aeruginosa*	AAC(3) + APH(3')-II	+	Bristol
4219	*P. aeruginosa*	AAC(3)-III	+	Schering (5)
4227	*P. aeruginosa*	AAC(3)-Ia	+	Schering
744	*Escherichia coli*(Tn*1699*)	AAC(3)-I + APH(3')-I	−	C. Rubens (42)
746	*E. coli*	AAC(3)-III	−	C. Rubens
898	*Klebsiella pneumoniae*	AAC(3)-I	−	Schering
1680	*Acinetobacter anitratus*	AAC(3)-I + APH(3')-I	−	Bristol
4231	*Salmonella enteritidis*	AAC(3)-IV	−	Schering (8)
4233	*Klebsiella pneumoniae*	AAC(3)-V	−	Schering (56)
4236	*Serratia marcescens*	AAC(3)-V	−	Schering (29)

[a] The probe used was the *Ava*I-*Eco*RV fragment of pFCT4374.
[b] See Table 1, footnote *b*.

one *E. coli* and one *Klebsiella pneumoniae*, each classified as an AAC(3)-I-containing strain, were positive with the probe. Finally, the DNAs from an additional 219 Gm^r and 58 Gm^s gram-negative bacilli isolated from patients at the Seattle Veterans Administration Medical Center were tested with the probe (Table 4). The DNA from four isolates, two *K. pneumoniae* and two *Serratia marcescens*, all demonstrating the AAC(3)-I phenotype, demonstrated homology with the probe. Although obtained from four different patients, the two *K. pneumoniae* strains were shown to be

TABLE 3. Hybridization of *aacC1* probe with DNA from enzymatically defined clinical isolates[a]

Strain	Organism	Enzyme	*aacC1* probe hybridization[b]	Source
3780	*Escherichia coli*	AAC(3)-I	+	Urine
3846	*Klebsiella pneumoniae*	AAC(3)-I	+	Urine
449	*Providencia stuartii*	AAC(2')	−	Urine
923	*P. stuartii*	AAC(2')	−	Urine
3593	*P. stuartii*	AAC(2')	−	Urine
3940	*P. stuartii*	AAC(2')	−	Urine
74	*Serratia marcescens*	AAC(3)-III	−	Urine
3598	*Enterobacter cloacae*	AAC(3)-V	−	Urine
3592	*Proteus mirabilis*	AAC(3)-V + AAC(6')	−	Urine
4043	*Providencia stuartii*	AAC(3)-V + AAC(6')	−	Urine
3658	*Citrobacter diversus*	AAD(2")	−	Urine
3692	*C. diversus*	AAD(2")	−	Urine
3642	*Escherichia coli*	AAD(2")	−	Urine
3834	*E. coli*	AAD(2")	−	Urine
4035	*E. coli*	AAD(2")	−	Urine
3781	*Klebsiella oxytoca*	AAD(2")	−	Urine
3933	*K. oxytoca*	AAD(2")	−	Urine
3833	*Klebsiella pneumoniae*	AAD(2")	−	Urine
3152	*Proteus mirabilis*	AAD(2")	−	Wound
3571	*Serratia marcescens*	AAD(2")	−	Sputum
3689	*Escherichia coli*	AAD(2")	−	Urine
3966	*E. coli*	AAD(2")	−	Urine
3775	*Morganella morganii*	AAD(2")	−	Urine
3058	*Serratia marcescens*	AAD(2")	−	Urine
1490	*Escherichia coli*	APH(3')-III	−	Urine

[a] From the Seattle Veterans Administration Medical Center.
[b] The probe used was the *Ava*I-*Eco*RV fragment of pFCT4374.

similar by plasmid fingerprinting, while the two *S. marcescens* strains appeared to be different isolates (data not shown). None of the gentamicin-susceptible strains demonstrated homology with the probe.

DISCUSSION

We have cloned and determined the DNA sequence of an AAC(3)-I gene (*aacC1*) from the enteric plasmid pJR88. The amino-terminal sequence of the AAC(3)-I protein, previously determined by Hsiang et al. (26), was helpful in determining the initiation site of the protein. DNA sequence homology was noted with a variety of other resistance

TABLE 4. Hybridization of *aacC1* probe with DNA from gentamicin-resistant and -susceptible clinical isolates[a]

Organism	No. tested	No. positive
Gentamicin resistant	219	4
Klebsiella pneumoniae	64	2
Escherichia coli	58	0
Providencia stuartii	26	0
Citrobacter diversus	23	0
Serratia marcescens	21	2
Citrobacter freundii	6	0
Morganella morganii	5	0
Klebsiella oxytoca	4	0
Proteus mirabilis	3	0
Proteus rettgeri	3	0
Enterobacter aerogenes	2	0
Enterobacter cloacae	2	0
Providencia rettgeri	1	0
Klebsiella ozaenae	1	0
Gentamicin susceptible	58	
Escherichia coli	16	0
Proteus mirabilis	15	0
Citrobacter diversus	4	0
Klebsiella pneumoniae	4	0
Providencia rettgeri	4	0
Serratia marcescens	4	0
Morganella morganii	3	0
Providencia stuartii	3	0
Proteus rettgeri	2	0
Enterobacter cloacae	1	0
Klebsiella oxytoca	1	0
Klebsiella ozaenae	1	0

[a] From Seattle Veterans Administration Medical Center. The probe used was the *Ava*I-*Eco*RV fragment of pFCT4374.

determinants, including several β-lactamase, dihydrofolate reductase, and aminoglycoside resistance genes, both at the 5' and 3' ends of the ORF of the *aacCl* gene. This is consistent with the observations of Cameron et al. (12) and Ouellette et al. (41), who noted areas of homology between the *aadB* gene and the OXA-1 gene, respectively, and several other resistance determinants.

In our previous report of the sequence of the *aacAl* gene (54), we demonstrated by a series of deletion experiments that the −35 and −10 sequences used by this determinant were probably different from those described by Cameron et al. (12). The unique feature of the *aacAl* gene was the insertion of three guanine residues which resulted in an optimal spacing of 17 bp between potential −35 and −10 sequences. Our analysis of the 5'-flanking region of *aacCl* revealed that several other genes, previously reported to use the −35 and −10 regions of Cameron et al., have the same insert. We suggest that this alternate promoter region is also used by the OXA-1 gene on RGN238 and the *aadA* gene from R538-1. Deletion studies similar to those undertaken for the *aacAl* gene will be necessary to confirm this, however.

We also noted that the restriction map of the *aacCl* gene and surrounding regions is consistent with areas of the Tn*21* family of transposons as described by Schmidt (45) and Kratz et al. (31). The map of the pFCT3111 insert is similar to the region between the *Eco*RI sites at positions 11 and 16 on the map of Kratz et al. (see Fig. 1, panel C of reference 31), although our fragment shows two insertions in this area. The first insertion is the *aacCl* gene, and the second does not produce a detectable phenotype. The presence of sulfonamide and streptomycin resistance genes on the cloned fragment in pFCT3111, in addition to the presence of a mercury resistance gene on pJR88, supports the presence of a Tn*21*-like element on pJR88. However, the ability of the *aacCl* gene to transpose from pJR88 to another replicon has not been investigated.

With regard to probe development, our initial attempts to prepare a restriction fragment probe for the *aacCl* gene before determining the nucleotide sequence were unsuccessful. Each restriction fragment probe we tested demonstrated homology with plasmid DNA encoding aminoglycoside resistance genes other than *aacCl*. The DNA sequencing data from the *aacCl* gene revealed that those probes extended beyond the boundaries of the AAC(3)-I ORF or included the first 59 bp of the ORF of *aacCl*. This reinforces the benefits of developing probes from known nucleotide sequences.

To test the specificity of the *aacCl* probe, it was necessary to assemble a series of genetically defined strains containing known aminoglycoside resistance genes. From our studies with these organisms, and particularly with the so-called enzymatically defined isolates, it was clear that probes are superior to the MIC-typing method and phosphocellulose paper-binding assay for classifying aminoglycoside resistance genes in bacterial isolates. While the MIC method of Miller et al. (37) is more reliable in our estimation than the phosphocellulose paper-binding assay, it is limited by its inability to detect and classify phosphotransferases and the inability to detect certain combinations of enzymes, such as AAC(2') in the presence of an *aadB* gene (46). The development of additional probes for aminoglycoside, β-lactamase, and trimethoprim resistance genes must be undertaken cautiously so as not to include the flanking sequences that are so commonly present, particularly among genes involved in the Tn*21* family of elements. It was for this reason that we chose to determine the DNA sequence of the *aacCl* gene.

Several studies using traditional methods have attempted to delineate the presence of various resistance genes in clinical isolates of bacteria (16, 29, 37, 46). These studies, while important, have been hampered by the limitations of the techniques employed. More recent studies using batteries of DNA probes to various resistance genes demonstrate the value of hybridization assays for screening large numbers of organisms quickly and efficiently, limited only by the numbers of probes employed (2, 28, 47, 52). Additional probe studies will help us to understand the epidemiology of resistance genes, how genes disseminate in nature, and the role of transposons, such as Tn*21*, in this process.

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