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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

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The gene specifying the bifunctional 6′-aminoglycoside acetyltransferase [AAC(6′)] 2″-aminoglycoside phosphotransferase [APH(2″)] enzyme from the *Streptococcus faecalis* plasmid pIP800 was cloned in *Escherichia coli*. A single protein with an apparent molecular weight of 56,000 was specified by this cloned determinant as detected in minicell experiments. Nucleotide sequence analysis revealed the presence of an open reading frame capable of specifying a protein of 479 amino acids and with a molecular weight of 56,850. The deduced amino acid sequence of the bifunctional AAC(6′)-APH(2″) gene product possessed two regions of homology with other sequenced resistance proteins. The N-terminal region contained a sequence that was homologous to the chloramphenicol acetyltransferase of *Bacillus pumilus*, and the C-terminal region contained a sequence homologous to the aminoglycoside phosphotransferase of *Streptomyces fradiae*. Subcloning experiments were performed with the AAC(6′)-APH(2″) resistance determinant, and it was possible to obtain gene segments independently specifying the acetyltransferase and phosphotransferase activities. These data suggest that the gene specifying the AAC(6′)-APH(2″) resistance enzyme arose as a result of a gene fusion.

Resistance to aminoglycoside-aminocylitol antibiotics among both gram-positive and gram-negative bacteria occurs primarily as a result of plasmid- or transposon-encoded enzymes that modify the antibiotics. Three classes of enzymes have been identified: aminoglycoside acetyltransferases (AACs), adenylyltransferases, and phosphotransferases (APHs). A single enzyme frequently modifies several antibiotics, with the same modification mechanism used for each antibiotic. An exception to this characteristic is the bifunctional resistance enzyme found in strains of streptococci (10) and staphylococci (16–18), which modifies both gentamicin and kanamycin. This bifunctional resistance enzyme possesses both 6′-acetyltransferase [AAC(6′)] and 2″-phosphotransferase [APH(2″)] activities, and attempts to separate the two activities by conventional protein separation techniques have not been successful (10, 16).

Recently, Ubukata et al. (35) purified a bifunctional AAC(6′)-APH(2″) enzyme from *Staphylococcus aureus* and determined that it had a molecular weight of 56,000. Sequencing data available for a number of acetyltransferases (2, 8, 14, 15, 31) and phosphotransferases (3, 13, 26, 33, 34) reveal that the average molecular weight for these enzymes is about 30,000, approximately one-half the size of the bifunctional resistance enzyme isolated from *S. aureus*. These observations, along with the knowledge that plasmids are subjected to frequent rearrangements and changes (9), led to the speculation that the AAC(6′)-APH(2″) resistance determinant arose as a gene fusion product of two individual resistance determinants. The availability of a cloned AAC(6′)-APH(2″) determinant from streptococci (4) made possible the nucleotide sequencing of this gene, which we report in this communication.

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MATERIALS AND METHODS

Bacteria and media. The bacterial strains and plasmids used in this study are listed in Table 1. All streptococcal strains were grown in brain heart infusion broth. *Escherichia coli* JM109 was used as the recipient for transfection experiments with M13 bacteriophage vectors (38) and was routinely grown in 2× YT broth (24).

Enzymes and chemicals. Restriction enzymes, T4 DNA ligase, the Klenow fragment of DNA polymerase I, and the M13 15-base primer were purchased from Bethesda Research Laboratories, Inc., and were used in accordance with the specifications of the manufacturer. Deoxy and dideoxy nucleotide triphosphates were purchased from P-L Biochemicals, Inc., and [α-^{32}P]dATP was purchased from Amersham Corp. [^{35}S]methionine was purchased from New England Nuclear Corp.

Determination of MICs. *E. coli* JM109 containing plasmid pUC8, pSF815A, or pSF915A was inoculated into sterile microdilution plates containing 2× YT media with appropriate serial dilutions of gentamicin (concentration range, 8 to 1,024 μg/ml). After overnight incubation, the lowest concentration of antibiotic that caused complete inhibition of growth was the MIC.

TABLE 1. Bacterial strains and plasmids

Strain	Plasmid and phenotype	Derivation or reference
S. faecalis JH-102	pIP800 Km Gm Cm	10
S. sanguis SM3013	pGB3013 MLS,[a] Km Gm Cm	4
E. coli		
P678-54		1
JM109	pUC8 Ap	38
SF978	pSF978 Km Gm Ap	pUC9 containing 7.8-kb fragment from pGB3013
SF815A	pSF815A Km Gm Ap	pUC8 containing 1.5-kb *Alu*I fragment from pSF978
SF915A	pSF915A Km Gm Ap	pUC9 containing 1.5-kb *Alu*I fragment from pSF815A
SF815AC	pSF815AC Km Ap	pUC8 containing 781-bp *Alu*I-*Sca*I fragment from pSF815A
SF815AP	pSF815AP Ap	pUC8 containing 767-bp *Sca*I-*Alu*I fragment from pSF815A
SF940	pSF940 Gm Ap	pUC8 containing 1,045-bp fragment from pSF815A

[a] MLS, Resistance to macrolides-lincosamides-streptogramin B.

Enzyme assays. Assays for AAC(6′) and APH(2″) activities were performed as previously described (10).

Minicell analysis of plasmid-encoded proteins. *E. coli* P678-54 was used to produce minicells (1). The preparation of competent cells and the procedure for transformation of plasmid pUC8 and its derivatives was done as previously described (21). The purification of minicells, labeling of plasmid-encoded proteins, and autoradiography were done as described by Stoker et al. (32).

M13 subcloning and nucleotide sequencing. The gene specifying the bifunctional AAC(6′)-APH(2″) resistance enzyme was obtained by cloning a 7.8-kilobase (kb) *Bsp*RI-*Hpa*II fragment from the streptococcal plasmid pGB3013. Subsequently, a 1.5-kb *Alu*I fragment was subcloned into the *Sma*I site of pUC8 to form the plasmid pSF815A. This plasmid served as a source for obtaining large amounts of the 1.5-kb fragment, which was unidirectionally degraded with *Bal* 31 by a modification of the procedure described by Gilmore et al. (12). Briefly, 5 mg of pSF815 was digested with *Eco*RI and *Pst*I, polylinker sites adjacent to the 1.5-kb cloned fragment, and this fragment was isolated by centrifugation through a 10 to 40% sucrose gradient (22). The 1.5-kb fragment was self-ligated in the presence of T4 DNA ligase to produce long polymers. These polymers were subsequently digested with *Pst*I to produce dimers of the 1.5-kb fragment linked at the *Eco*RI site. The dimers were digested with *Bal* 31, and at 5-min intervals, samples were removed and the *Bal* 31-digested DNA was precipitated with ethanol. This DNA was further digested with *Eco*RI to produce fragments containing *Eco*RI and *Bal* 31 ends. Finally, the DNA was ligated into *Sma*I-*Eco*RI-digested mp18 and transfected into *E. coli* JM109. Single-stranded recombinant phage template DNA for use in sequencing was prepared as previously described (29).

Sequencing reactions were performed by the Sanger dideoxy chain termination method (30) by using the procedures described by Amersham. All sequences were confirmed from at least two overlapping clones, and the entire AAC(6′)-APH(2″) resistance gene sequence was determined on both strands. The sequence information was analyzed by the James M. Pustell DNA/protein sequencing program obtained from International Biotechnologies, Inc.

RESULTS

Subcloning of the AAC(6′)-APH(2″) resistance determinant. The streptococcal plasmid pGB3013 served as the source of the gene specifying the bifunctional AAC(6′)-APH(2″) enzyme (4), and a 7.8-kb *Bsp*RI-*Hpa*II fragment was cloned into *Sma*I-*Acc*I-digested pUC9 to form the recombinant plasmid pSF978. Transformation of this plasmid into *E. coli* resulted in expression of both the kanamycin and gentamicin resistance phenotypes. Subsequent subcloning was accomplished by introduction of a 1.5-kb *Alu*I fragment obtained from pSF978 into the *Sma*I site of pUC9 to form the recombinant plasmid pSF915A. The orientation of this fragment was reversed by *Eco*RI-*Bam*HI digestion and subsequent ligation of the 1.5-kb fragment into similarly cut pUC8 to form the recombinant plasmid pSF815A. The recombinant plasmids contained the AAC(6′)-APH(2″) determinant in both orientations and expressed resistance to both gentamicin and kanamycin in *E. coli*. Determination of the MICs of gentamicin for the *E. coli* strains containing plasmids with

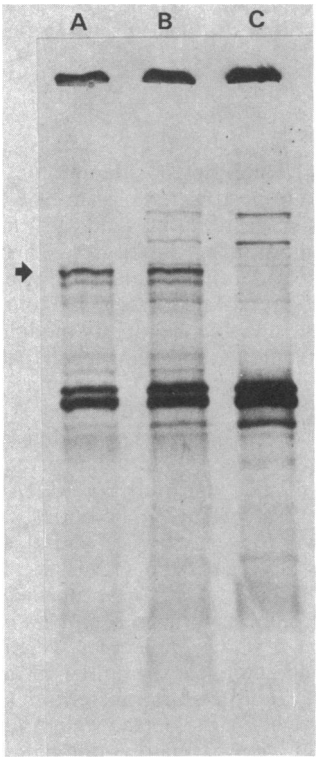

FIG. 1. Autoradiograph of [^{35}S]methionine-labeled polypeptides synthesized in *E. coli* containing the following plasmids: pSF815A (lane A), pSF915A (lane B), and pUC8 (lane C). An apparent molecular weight of 56,000 (arrow) for the AAC(6′)-APH(2″) resistance determinant was determined by comparison with the following proteins (molecular weight): bovine serum albumin (66,200), egg albumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and lysozyme (14,400).

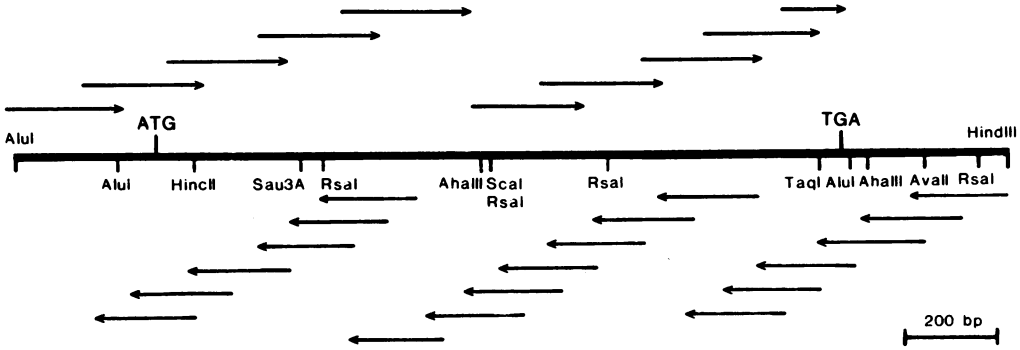

FIG. 2. Sequencing strategy and partial restriction map of the AAC(6')-APH(2") resistance determinant, which is contained in a 2,120-bp fragment with a 5' *Alu*I site and a 3' *Hin*dIII site. The arrows indicate the direction and extent of the sequence derived from each independent clone.

the resistance determinant in both orientations was performed. The MIC for the *E. coli* strain containing plasmid pSF815A increased in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG) (256 μg/ml) and decreased in the presence of glucose (32 μg/ml), indicating that expression of the AAC(6')-APH(2") resistance determinant was under the influence of the *lacZ* gene promoter. The MIC for the *E. coli* strain containing plasmid pSF915A was constant in the presence of either IPTG or glucose (32 μg/ml), suggesting that the insert in the opposite orientation possessed its own promoter and was not influenced by the *lacZ* gene promoter.

A similar approach of cloning the 1.5-kb *Alu*I fragment from the original pIP800 plasmid into pUC8 resulted in the expression of resistance to both antibiotics in *E. coli*, and the insert was identical to plasmid pSF815A described above, as judged by restriction mapping. This approach was necessary to eliminate the possibility that gene fusions had occurred during previous cloning experiments in *Streptococcus sanguis* (4), an organism in which gene rearrangements are known to occur after transformation (5, 19, 20).

Minicell detection of the bifunctional resistance gene product. To detect the protein specified by the cloned AAC(6')-APH(2") resistance determinant, plasmid-encoded proteins were analyzed by using the *E. coli* minicell system (32). Minicells containing plasmids with the AAC(6')-APH(2") resistance gene were labeled with [^{35}S]methionine, and the lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography. The major protein produced by the clones containing the AAC(6')-APH(2") resistance determinant had an apparent molecular weight of 56,000 (Fig. 1).

Nucleotide sequence of the AAC(6')-APH(2") resistance gene. The sequencing strategy and restriction map for the AAC(6')-APH(2") resistance gene is depicted in Fig. 2. The sequence of the entire 1.5-kb *Alu*I fragment containing the AAC(6')-APH(2") resistance determinant and flanking regions (Fig. 3) was determined by the method of Sanger et al. (30). In all, 2,120 base pairs (bp) spanned a segment with a 5' *Alu*I site and a 3' *Hin*dIII site. The overlapping array of fragments produced by unidirectional digestions with *Bal* 31 provided sequence duplication from both strands for each region.

An open reading frame containing 1,437 bp codes for the AAC(6')-APH(2") resistance protein. The deduced protein which starts at the ATG codon at positions 304 to 306 and extends to the termination codon TGA at positions 1741 to 1743 contains 479 amino acids and has a molecular weight of 56,850. A putative ribosome-binding site sequence, AG GTGAT, is located six nucleotides upstream from the translation initiation codon. Further upstream are sequences similar to a Pribnow box centered at position −10 (TATAAAG) and an RNA polymerase recognition site at position −35 (ATGAAA). Downstream of the coding region is a 10-bp palindromic sequence adjacent to the TGA termination codon.

Amino acid sequence and protein homology. A comparison of the deduced amino acid sequence of the AAC(6')-APH(2") resistance gene product with other known sequenced proteins revealed two interesting partial homologies (Fig. 4). In the N-terminal region between positions 96 and 223, there are 30 amino acids which are identical and 52 which are similar to those of the chloramphenicol acetyltransferase specified by the *cat-86* gene of *Bacillus pumilus* (14). In the C-terminal region between positions 352 and 448, there are 28 amino acids which are identical and 42 which are similar to those of the aminoglycoside APH(3') of *Streptomyces fradiae* (33). Although these homologies are only partial, it is possible to tentatively assign the AAC(6') activity to the N-terminal region and the APH(2") activity to the C-terminal region of the bifunctional resistance gene product. These assignments are consistent with the origin of the bifunctional resistance protein as a fusion between two individual genes. Such a hypothesis was directly testable by cloning the individual regions and selecting for individual resistance phenotypes.

Subcloning of gene regions specifying AAC(6') or APH(2") activities. A single *Sca*I site is located in the sequence between the putative domains and facilitated the subcloning of each gene segment. The strategy for the subcloning of each gene segment into plasmid pUC8 is presented in Fig. 5. The recombinant plasmid pSF815AC contained the 781-bp *Alu*I-*Sca*I fragment and specified only AAC(2") activity. The recombinant plasmid pSF815AP contained a 767-bp *Sca*I-*Alu*I fragment, and this gene segment was expected to contain an in-frame fusion with the *lacZ* gene and express APH(2") activity under the control of the *lacZ* promoter. However, there was no expression of either resistance phenotype by this clone.

To further explore the possibility of obtaining a region which expressed only APH(2") activity, a number of clones containing deletions in the 5' region of the 1.5-kb *Alu*I fragment were tested for both AAC(6') and APH(2") activities. One clone containing plasmid pSF940AP and missing the region specifying the N-terminal 137 amino acids and approximately one-half of the region homologous to other acetyltransferase proteins specified only APH(2") activity.

```
5'
AGCTAAAGAAAATAATAAAATTATAGGATTTGCATATTGCTATACACTTTAAGACCTGATGGAAAAACAATGTTTTATTTACACTCAATAGGAATGTTAC
                                                  50                                                  100

CTAACTATCAAGACAAAGGTTATGGTTCAAAATTATTATCTTTTATTAAGGAATATTCTAAAGAGATTGGTTGTTCTGAAATGTTTTTAATAACTGATAA
                                                 150                                                 200

AGGTAATCCTAGAGCTTGCCATGTATATGAAAAATTAGGTGGTAAAAATGATTATAAAGATGAAATAGTATATGTATATGATTATGAAAAAGGTGATAAA
                                                 250                                                 RBS 300
                                          10                                                  20
TAA ATG AAT ATA GTT GAA AAT GAA ATA TGT ATA AGA ACT TTA ATA GAT GAT GAT TTT CCT TTG ATG TTA AAA TGG TTA
    Met Asn Ile Val Glu Asn Glu Ile Cys Ile Arg Thr Leu Ile Asp Asp Asp Phe Pro Leu Met Leu Lys Trp Leu

                30                                                  40                                  50
ACT GAT GAA AGA GTA TTA GAA TTT TAT GGT GGT AGA GAT AAA AAA TAT ACA TTA GAA TCA TTA AAA AAA CAT TAT ACA
Thr Asp Glu Arg Val Leu Glu Phe Tyr Gly Gly Arg Asp Lys Lys Tyr Thr Leu Glu Ser Leu Lys Lys His Tyr Thr

                                    60                                                  70
GAG CCT TGG GAA GAT GAA GTT TTT AGA GTA ATT ATT GAA TAT AAC AAT GTT CCT ATT GGA TAT GGA CAA ATA TAT AAA
Glu Pro Trp Glu Asp Glu Val Phe Arg Val Ile Ile Glu Thr Asn Asn Val Pro Ile Gly Tyr Gly Gln Ile Tyr Lys

        80                                                  90                                  100
ATG TAT GAT GAG TTA TAT ACT GAT TAT CAT TAT CCA AAA ACT GAT GAG ATA GTC TAT GGT ATG GAT CAA TTT ATA GGA
Met Tyr Asp Glu Leu Tyr The Asp Tyr His Tyr Pro Lys Thr Asp Glu Ile Val Tyr Gly Met Asp Gln Phe Ile Gly

                        110                                                 120
GAG CCA AAT TAT TGG AGT AAA GGA ATT GGT ACA AGA TAT ATT AAA TTG ATT TTT GAA TTT TTG AAA AAA GAA AGA AAT
Glu Pro Asn Tyr Trp Ser Lys Gly Ile Gly Thr Arg Tyr Ile Lys Leu Ile Phe Glu Phe Leu Lys Lys Glu Arg Asn

130                                 140                                                 150
GCT AAT GCA GTT ATT TTA GAC CCT CAT AAA AAT AAT CCA AGA GCA ATA AGG GCA TAC CAA AAA TCT GGT TTT AGA ATT
Ala Asn Ala Val Ile Leu Asp Pro His Lys Asn Asn Pro Arg Ala Ile Arg Ala Tyr Gln Lys Ser Gly Phe Arg Ile

                160                                                 170                                 180
ATT GAA GAT TTG CCA GAA CAT GAA TTA CAC GAG GGC AAA AAA GAA GAT TGT TAT TTA ATG GAA TAT AGA TAT GAT GAT
Ile Glu Asp Leu Pro Glu His Glu Leu His Glu Gly Lys Lys Glu Asp Cys Tyr Leu Met Glu Tyr Arg Tyr Asp Asp

                                    190                                                 200
AAT GCC ACA AAT GTT AAG GCA ATG AAA TAT TTA ATT GAG CAT TAC TTT GAT AAT TTC AAA GTA GAT AGT ATT GAA ATA
Asn Ala Thr Asn Val Lys Ala Met Lys Tyr Leu Ile Glu His Tyr Phe Asp Asn Phe Lys Val Asp Ser Ile Glu Ile

        210                                                 220                                 230
ATC GGT AGT GGT TAT GAT AGT GTG GCA TAT TTA GTT AAT AAT GAA TAC ATT TTT AAA ACA AAA TTT AGT ACT AAT AAG
Ile Gly Ser Gly Tyr Asp Ser Val Ala Tyr Leu Val Asn Asn Glu Tyr Ile Phe Lys Thr Lys Phe Ser Thr Asn Lys

                        240                                                 250
AAA AAA GGT TAT GCA AAA GAA AAA GCA ATA TAT AAT TTT TTA AAT ACA AAT TTA GAA ACT AAT GTA AAA ATT CCT AAT
Lys Lys Gly Try Ala Lys Glu Lys Ala Ile Tyr Asn Phe Leu Asn Thr Asn Leu Glu Thr Asn Val Lys Ile Pro Asn

260                                 270                                                 280
ATT GAA TAT TCG TAT ATT AGT GAT GAA TTA TCT ATA CTA GGT TAT AAA GAA ATT AAA GGA ACT TTT TTA ACA CCA GAA
Ile Glu Tyr Ser Tyr Ile Ser Asp Glu Leu Ser Ile Leu Gly Tyr Lys Glu Ile Lys Gly Thr Phe Leu Thr Pro Glu

                290                                                 300                                 310
ATT TAT TCT ACT ATG TCA GAA GAA GAA CAA AAT TTG TTA AAA CGA GAT ATT GCC AGT TTT TTA AGA CAA ATG CAC GGT
Ile Try Ser Thr Met Ser Glu Glu Glu Gln Asn Leu Leu Lys Arg Asp Ile Ala Ser Phe Leu Arg Gln Met His Gly

                                    320                                                 330
TTA GAT TAT ACA GAT ATT AGT GAA TGT ACT ATT GAT AAT AAA CAA AAT GTA TTA GAA GAG TAT ATA TTG TTG CGT GAA
Leu Asp Try Thr Asp Ile Ser Glu Cys Thr Ile Asp Asn Lys Gln Asn Val Leu Glu Glu Tyr Ile Leu Leu Arg Glu
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       340                                 350                                 360
ACT ATT TAT AAT GAT TTA ACT GAT ATA GAA AAA GAT TAT ATA GAA AGT TTT ATG GAA AGA CTA AAT GCA ACA ACA GTT
Thr Ile Tyr Asn Asp Leu Thr Asp Ile Glu Lys Asp Tyr Ile Glu Ser Phe Met Glu Arg Leu Asn Ala Thr Thr Val

                    370                                 380
TTT GAG GGT AAA AAG TGT TTA TGC CAT AAT GAT TTT AGT TGT AAT CAT CTA TTG TTA GAT GGC AAT AAT AGA TTA ACT
Phe Glu Glu Lys Lys Cys Leu Cys His Asn Asp Phe Ser Cys Asn His Leu Leu Leu Asp Gly Asn Asn Arg Leu Thr

390                                 400                                 410
GGA ATA ATT GAT TTT GGA GAT TCT GGA ATT ATA GAT GAA TAT TGT GAT TTT ATA TAC TTA CTT GAA GAT AGT GAA GAA
Gly Ile Ile Asp Phe Gly Asp Ser Gly Ile Ile Asp Glu Tyr Cys Asp Phe Ile Tyr Leu Leu Glu Asp Ser Glu Glu

          420                                 430                                 440
GAA ATA GGA ACA AAT TTT GGA GAA GAT ATA TTA AGA ATG TAT GGA AAT ATA GAT ATT GAG AAA GCA AAA GAA TAT CAA
Glu Ile Gly Thr Asn Phe Gly Glu Asp Ile Leu Arg Met Tyr Gly Asn Ile Asp Ile Glu Lys Ala Lys Glu Tyr Gln

                    450                                 460
GAT ATA GTT GAA GAA TAT TAT CCT ATT GAA ACT ATT GTT TAT GGA ATT AAA AAT ATT AAA CAG GAA TTT ATC GAA AAT
Asp Ile Val Glu Glu Tyr Tyr Pro Ile Glu Thr Ile Val Tyr Gly Ile Lys Asn Ile Lys Gln Glu Phe Ile Glu Asn

       470
GGT AGA AAA GAA ATT TAT AAA AGG ACT TAT AAA GAT TGA TTATATAATATATGAAAAGCTATTATAAAAGACATTAGTATTAAATAGTTT
Gly Arg Lys Glu Ile Tyr Lys Arg Thr Tyr Lys Asp  *

AAAAAAATGAAAAATAATAAAGGAAGTGAGTCAAGTCCAGACTCCTGTGTAAAATGCTATACAATGTTTTTACCATTTCTACTTATCAAAATTGATGTAT
 1800                                1850

TTTCTTGAAGAATAAATCCATTCATCATGTAGGTCCATAAGAACGGCTCCAATTAAGCGATTGGCTGATGTTTGATTGGGGAAGATGCGAATAATCTTTT
 1900                                1950

CTCTTCTGCGTACTTCTTGATTCAGTCGTTCAATTAGATTGGTACTCTTTAGTCGATTGTGGGAATTTCCTTGTACGGTATATTGAAAGGCGTCTTCGAA
 2000                                2050

TCCATCATCCAATGATGCGCAAGCTT 3'
 2100               2120
```

FIG. 3. Nucleotide sequence of the AAC(6')-APH(2") resistance determinant and flanking regions. Numbering begins at the 5' end of the sequence. The deduced amino acid sequence of the bifunctional resistance protein is given below the nucleotide sequence. Putative promoter and ribosome-binding site (RBS) sequences are underlined, as is an inverted repeat sequence adjacent to the TGA termination codon.

Thus, the APH(2") activity can exist in the absence of the AAC(6') activity.

DISCUSSION

The AAC(6')-APH(2") resistance determinant from the *Streptococcus faecalis* plasmid pIP800 (10) was cloned previously in *S. sanguis* (4) and was cloned in this study in *E. coli*. Minicell experiments showed that the cloned determinant specified a single protein with an apparent molecular weight of 56,000. Nucleotide sequence analysis revealed the presence of an open reading frame capable of specifying a protein of 479 amino acids and with a molecular weight of 56,850. These results are in agreement with the model proposed by Le Goffic et al. (18) of one polyfunctional enzyme for two staphylococcal activities and the report of Ubukata et al. (35), who recently purified an AAC(6')-APH(2") bifunctional enzyme from *S. aureus* which had a molecular weight of 56,000.

A number of sequences were identified that were similar to transcription and translation sequences present in other gram-positive organisms. For example, a putative ribosome-binding site is located 7 bp upstream from the ATG initiation codon. The −10 sequence (Pribnow box) TATAAAG which centers from the putative mRNA start point (A-265) varies from the *E. coli* consensus promoter sequence TATAATG by a single nucleotide and is similar to sequences reported for bacilli (7, 23, 25) and staphylococci (28). The putative −35 sequence ATGAAA varies from the *E. coli* sequence by 2 bp and is similar to sequences reported for bacilli (23, 25, 27) and streptococci (34).

The deduced amino acid sequence of the AAC(6')-APH(2") gene product contains two regions of homology with other sequenced resistance proteins. In the N-terminal region, 82 of 126 amino acids are identical or similar to those of the chloramphenicol acetyltransferase specified by the *cat-86* gene of *B. pumilus* (14). Two other chloramphenicol acetyltransferase genes have been sequenced, and whereas the proteins encoded by these genes have extensive homology with each other (15, 31), the greatest homology of the bifunctional resistance protein is with the *cat-86*-specified gene product. Interestingly, there was little homology with the proteins specified by three *E. coli* aminoglycoside AACs (2).

```
AAC/APH   1 MNIVENEICIRTLIDDDFPLMLKWLTDERVLEFYGGRDKKYTLESLKKHYTEPWEDEVFR

cat-86   31                                    * MDQ  *E *YW        **
AAC/APH  61 VIIEYNNVPIGYGQIYKMYDELYTDYHYPKTDEIVYGMDQFIGEPNYWSKGIGTRYIKLI

cat-86   85 ***L*KE ** * I *P **N **  ***  * **F*  **L P*****E** * * L
AAC/APH 121 FEFLKKERNANAVILDPHKNN-PRAIRAY--QKSGFRIIEDL-PEHELHEGKKEDCYLME

cat-86  145   **D * NV**   YL*  * ***FKV** *II *    V * V **Y
AAC/APH 177 YRYDDNATNVKA-MKYLIEHY-FDNFKVDSIEIIGSGYDSVAYLVNNEYIFKTKFSTNKK


AAC/APH 235 KGYAKEKAIYNFLNTNLETNVKIPNIEYSYISDELSILGYKEIKGTFLTPEIYSTMSEEE

APH     109                                                        * *
AAC/APH 295 QNLLKRDIASFLRQMHGLDYTDISECTIDNKQNVLEEYILLRETIYNDLTDIEKDYIESF

APH     169 ** L* T   ***  *CH*D** N**LLD G* R*TG*ID G  G* D * D*     *
AAC/APH 355 MERLNATTVFEGKKCLCHNDFSCNHLLLD-GNNRLTGIIDFGDSGIIDEYCDFIYLLED-

APH     231   *E*  *G****E *L *YG*  **K*K** ****E**
AAC/APH 413 --SEEE-IGTNFGEDILRMYGNIDIEKAKEYQDIVEEYYPIETIVYGIKNIKQEFIENGR

AAC/APH 470 KEIYKRTYKD
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FIG. 4. The alignment of predicted amino acid sequences of the AAC(6')-APH(2") bifunctional resistance protein (AAC/APH), the *cat-86* gene product from *B. pumilus* (14), and the APH gene product from *S. fradiae* (33). The sequences were aligned by the PRTALN program of Wilbur and Lipman (37). The gaps were introduced to achieve maximum alignment. Identical amino acids are shown with the corresponding one-letter amino acid symbols; an asterisk indicates similar amino acids. The numbering shown at the beginning of each amino acid sequence corresponds to that given by the original investigators.

In the C-terminal region of the bifunctional resistance protein, 70 of 96 amino acids are either identical or similar to the deduced APH protein of *S. fradiae* (33). The statistical significance of sequence similarities (37) was much higher for the deduced amino acid sequence of the APH(3') from *S. fradiae* than for the sequence of an APH from *E. coli* (3, 26) or streptococci (34). There was also some homology, albeit at a low significance level, to the deduced sequence of phosphoglucomutase. Such homologies may represent common sequences present at the catalytic sites of phosphorylating enzymes.

The possibility that a gene fusion was responsible for the contiguous protein containing AAC(6') and APH(2") activities was suggested by data available indicating that most AAC and APH resistance proteins have molecular weights of approximately 30,000. In addition, the sequence homology information from this study was consistent with separate domains for each resistance mechanism in the bifunctional

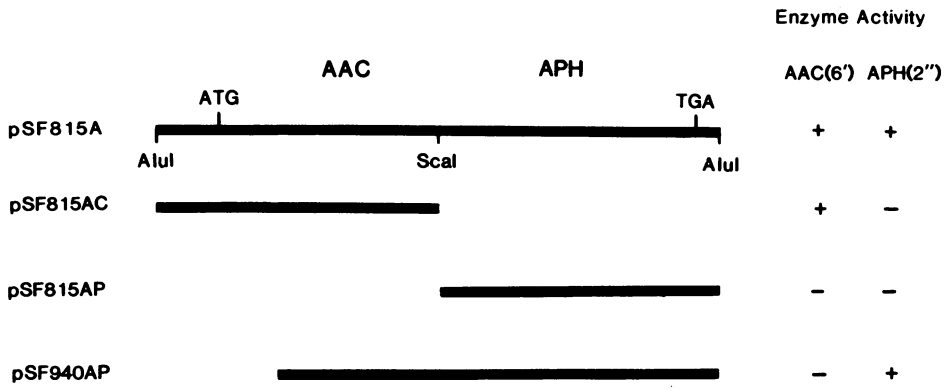

FIG. 5. Subcloning strategy for obtaining gene segments specifying individual AAC(6') and APH(2") activities. The recombinant plasmids designated on the left contain gene segments (indicated by the heavy line) cloned into the plasmid vector pUC8. Enzyme assays performed for each clone are indicated on the right.

resistance gene product. Further support for such an origin of the AAC(6')-APH(2") resistance determinant came from subcloning experiments in which each gene region could be obtained with expression of one activity and not the other. The 5' gene region, which specifies the N-terminal portion of the protein and AAC(6') activity, was obtained in a straightforward manner. Expression of the AAC(6') activity was possible because this gene region contained its own promoter and ribosome-binding sites, essential for transcription and translation.

Subcloning of the 3' region, which specifies the C-terminal portion of the bifunctional resistance protein and APH(2") activity, proved to be more problematic because this region did not contain its own promoter control region and because the APH(2") activity also results in a moderate level of resistance to kanamycin. Even though this gene region was subcloned and made in phase with the *lacZ* gene and under its promoter control, no APH activity or, in this case, resistance to gentamicin was observed. Whereas 120 additional amino acids are present upstream from the region of homology among APH proteins, this region may not be sufficient for activity or, alternatively, the new *lacZ*-APH(2") fused protein may be conformationally inactive. We did, however, obtain a gentamicin-resistant clone which was an in-frame fusion with the *lacZ* protein and under its control, which lacked the N-terminal 137 amino acids of the bifunctional resistance protein. This clone specified only APH(2") activity. Thus, the ability to obtain a gene segment specifying independently the AAC(6') and APH(2") activities is strong evidence in support of a gene-fusion origin of the AAC(6')-APH(2") resistance determinant.

Further speculation concerning the stage in evolution at which these determinants underwent gene fusion awaits further study. Evidence that there is more homology between the APH(2") region of the bifunctional resistance protein and that of the APH of *S. fradiae* than with similar determinants found in other gram-positive or gram-negative bacteria suggests that the fusion may have occurred between *Streptomyces* genes and was then passed on evolutionarily to bacteria. Such a proposal is consistent with the hypotheses of Walker and Walker (36) and Benveniste and Davies (6) that bacterial antibiotic resistance determinants may have originated in antibiotic-producing *Streptomyces* spp. The determination of the sequence of a *Streptomyces* AAC gene and its derived protein sequence should be of considerable interest in further assessing where the gene fusion event may have occurred. The possibility also exists that the AAC and APH genes may have fused after transfer to other bacteria because Dowding (11) has reported the electrophoretic separation of APH(2") and AAC(6') activities in *S. aureus*. Sequence information for these individual determinants could provide information about possible progenitorial forms of the bifunctional resistance determinant.

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