

New Mobile Gene Cassettes Containing an Aminoglycoside Resistance Gene, *aacA7*, and a Chloramphenicol Resistance Gene, *catB3*, in an Integron in pBWH301

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Received 25 August 1994/Returned for modification 2 November 1994/Accepted 4 January 1995

The multidrug resistance plasmid pBWH301 was shown to contain a *sull*-associated integron with five inserted gene cassettes, *aacA7-catB3-aadB-oxa2-orfD*, all of which can be mobilized by the integron-encoded DNA integrase. The *aadB*, *oxa2*, and *orfD* cassettes are identical to known cassettes. The *aacA7* gene encodes a protein that is a member of one of the three known families of aminoglycoside acetyltransferases classified as AAC(6′)-I. The chloramphenicol acetyltransferase encoded by the *catB3* gene is closely related to members of a recently identified family of chloramphenicol acetyltransferases. The *catB3* gene displays a relatively high degree of sequence identity to a chromosomally located open reading frame in *Pseudomonas aeruginosa*, and this may represent evidence for the acquisition by a cassette of a chromosomal gene.

pBWH301 is a 69-kb multidrug-resistant conjugative plasmid isolated from two bacterial species, *Enterobacter aerogenes* and *Enterobacter cloacae*, during an outbreak of amikacin resistance in Hospital Vargas in Venezuela (19). Most of the drug resistance genes in pBWH301 were found clustered within a 6.0-kb *Bam*HI fragment that confers resistance to the aminoglycosides amikacin (Ak^r), gentamicin (Gm^r), kanamycin (Km^r), netilmicin (Nt^r), and tobramycin (Tb^r), as well as resistance to chloramphenicol (Cm^r) and sulfonamides (Su^r). Two aminoglycoside resistance genes were identified on the basis of aminoglycoside resistance profiles: an *aacA* gene encoding a type I aminoglycoside acetyltransferase [AAC(6′)-I] that confers resistance to amikacin, netilmicin, and tobramycin and an *aadB* gene encoding an aminoglycoside adenyltransferase [AAD(2′)] that confers resistance to gentamicin, kanamycin, and tobramycin (19). A chloramphenicol resistance gene and a sulfonamide resistance gene were also identified. The presence of the restriction sites characteristic of the *sull* gene, which is normally found in the 3′-conserved segment of *sull*-associated integrons (37), suggested that the resistance genes may be integron associated (19).

The ability of bacteria to develop multiple-drug resistance is due in part to their ability to acquire new antibiotic resistance genes. Mobile elements called integrons determine a site-specific recombination system that is responsible for the acquisition of many antibiotic resistance determinants (14, 15, 37). A large number of antibiotic resistance genes (conferring resistance to aminoglycosides, β-lactams, chloramphenicol, and trimethoprim), as well as several unidentified open reading frames, have been found as inserts in integrons (13). These genes are contained in individual mobile units called gene cassettes that can be inserted into and excised from an integron by site-specific recombination (3–5, 13). Gene cassettes consist of a gene coding region (or open reading frame) and a recombination site known as a 59-base element which is located 3′ to the gene in the linear integrated form (4, 13, 14). The 59-base

elements vary in sequence and length but are all imperfect inverted repeats and are related to a consensus sequence at their outer ends (1, 5, 13). The 59-base elements play an essential role in the process of gene acquisition, because they are recognized by the integron-encoded DNA integrase (Int) (13, 27) and the recombination crossover occurs between the G and the first T of the GTT triplet of the seven-base core site found in each 59-base element at the end distal to the 3′ end of the gene (13, 30).

The integrons most commonly found in plasmids and transposons from clinical antibiotic-resistant isolates are associated with the *sull* Su^r determinant. These elements consist of a 5′-conserved segment that encodes Int and includes an Int-specific recombination site (*attI*), a 3′-conserved segment containing the *sull* gene, and a central region containing one or more integrated gene cassettes (14, 15, 32, 37). The cassettes generally do not include a promoter, but once cassettes are inserted into an integron, the genes are transcribed from a common promoter P_{ant} in the 5′-conserved segment (1, 6, 26, 37).

In this study, we have characterized the multidrug resistance region of pBWH301 by restriction mapping and DNA sequencing and confirmed the presence of an integron with typical 5′- and 3′-conserved segment regions. This integron was found to contain five inserted gene cassettes, *aacA7-catB3-aadB-oxa2-orfD*, all of which can be mobilized by Int. Two new cassettes containing the *aacA7* and *catB3* genes were identified. The AAC(6′)-I enzyme encoded by the *aacA7* gene is a member of one of the three known families of AAC(6′)-I enzymes (35). The *catB3* gene encodes a chloramphenicol acetyltransferase (CAT) that is a member of a recently identified family of CAT proteins (31, 39). The *catB* gene family includes three cassette-associated genes and a non-cassette-associated gene isolated from a chloramphenicol-resistant isolate of *Agrobacterium tumefaciens*. The *catB* genes share a significant level of DNA sequence identity with a chromosomally located open reading frame from *Pseudomonas aeruginosa*, and it is possible that this open reading frame represents an ancestor or a relative of the ancestor of the cassette-associated *catB* genes.

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TABLE 1. Plasmids used in this study

Plasmid	Description	Phenotype	Source or reference
pACYC184		Cm ^r Tc ^r	2
pJH213	6.0-kb <i>Bam</i> HI- <i>Bam</i> HI fragment from pBWH301 cloned into pUC19	Ak ^r Ap ^r Cm ^r Gm ^r Km ^r Nt ^r Su ^r Tb ^r	19
pSU2056	1.7-kb <i>Bam</i> HI- <i>Rsa</i> I fragment of Tn21 inserted into pUC19	Ap ^r Int ⁺	27
pMAQ50	pACYC184 with the Cm ^r gene inactivated	Tc ^r Cm ^s	This study
pMAQ105	4.6-kb <i>Bam</i> HI- <i>Hind</i> III fragment of pJH213 cloned into pMAQ50	Ak ^r Ap ^r Cm ^r Gm ^r Km ^r Nt ^r Tb ^r	This study

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used were *Escherichia coli* JM101 (Δ *lac-proAB*) *supE* *thi* F' (*traD36 proAB⁺ lacF^r lacZ Δ M15*) (44) as a host for M13 and DH5 α *supE44 Δ lacU169 (lacZ Δ M15) hsdR17 recA1 gyrA96 thi-1 relA1* as a host for plasmid constructions. Plasmids used in this study are listed in Table 1. Plasmid pMAQ50 was derived from the vector pACYC184 (2) by digestion with *Eco*RI, filling in the end with DNA polymerase (33), and religation. This procedure removed the *Eco*RI site and disrupted the pACYC184 chloramphenicol resistance gene (Table 1). A 4.6-kb *Bam*HI-*Hind*III fragment from pJH213 was isolated and cloned into *Bam*HI-*Hind*III-digested pMAQ50 to produce the plasmid pMAQ105. All plasmids were introduced into DH5 α either by transformation into cells made competent by the calcium chloride method (33) or by electroporation with a Gene-pulser (Bio-Rad, Richmond, Calif.).

Growth of cultures. Bacteria were cultured in Luria broth (28) supplemented with the appropriate antibiotics. Solid medium was Luria broth containing 1.5% agar (Amyl Media, Scoresby, Victoria, Australia). Antibiotic concentrations used routinely were 2.5 μ g of amikacin per ml, 20 or 50 μ g of ampicillin per ml, 10 μ g of chloramphenicol per ml, and 5 μ g of gentamicin per ml. All antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.). To determine the level of antibiotic resistance, plasmid-containing strains were streaked on gradient plates containing 0 to 200 μ g of one antibiotic per ml. With these results as a guide, a series of Luria broth agar plates containing a range of antibiotic concentrations were prepared and samples of overnight culture containing approximately 100 to 500 cells were plated in duplicate onto these plates. Colonies were counted after incubation at 37°C for 48 h, the percent survival was calculated, and the results of two independent determinations were averaged. The concentration of antibiotic required for 50% survival was then determined from a plot of the percent survival against the antibiotic concentration.

DNA procedures. Plasmid DNA was isolated with the Magic Miniprep kit (Promega, Madison, Wis.). DNA was digested with restriction enzymes according to the manufacturer's instructions, and the fragments were separated on 0.8% agarose gels. DNA fragments were purified from the agarose gels with a GeneClean II kit (Bio 101, Inc., La Jolla, Calif.) and cloned into appropriately digested pMAQ50 or M13mp18 and M13mp19. Sequencing was performed with double-stranded and single-stranded templates and a Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, Ohio) with synthetic primers. dITP replaced dGTP in all sequencing reactions. Primers were annealed to double-stranded template by one of two methods described by Jones and Schofield (22) and Hsiao (21). The DNA sequences of the *aacA7* and *catB3* genes were determined for both strands. The rest of the insert region and parts of the conserved segments were sequenced on one strand only. Primers were synthesized with a Pharmacia Gene Assembler Mark II. DNA sequences were compiled with the computer program RODENT (Pharmacia, Milwaukee, Wis.) and translated with the computer program NUMSEQ from the Cornell DNA sequence analysis package (12).

Cassette excision. Excision of gene cassettes from pMAQ105 (Table 1) was analyzed by propagating the plasmid in DH5 α cells which also contained pSU2056 (27), a recombinant plasmid which expresses the Int. DH5 α (pMAQ105, pSU2056) cells were grown overnight in Luria broth with no antibiotic selection. Plasmid DNA was isolated and transformed into DH5 α cells by electroporation under conditions in which the majority of cells received only one DNA molecule (4). Transformants were plated onto Luria broth plates containing one of the antibiotics amikacin (5 μ g/ml), chloramphenicol (10 μ g/ml), or gentamicin (5 μ g/ml) and screened for the presence of other antibiotic resistance genes by patching onto Luria broth plates containing the relevant antibiotic. Plasmid DNA was prepared from selected clones that were sensitive to one or more antibiotics and digested with *Bam*HI-*Hind*III, *Eco*RI, and *Xho*I to determine the number and order of cassettes. The new boundaries were sequenced with double-stranded template and the appropriate primers. The following primers were used: HS89 in the 5'-conserved segment 5' GGCTTGTTATGACTGT 3', bases 1303 to 1318 in reference 16; HS110 in *aacA7* 5' GGCTTAACCTCAGGCGT 3', bases 875 to 890 in Fig. 2; HS99 in *catB3* 5' TGTACCGCCCACTTG 3', complement of bases 994 to 1008 in Fig. 2; HS103 in *catB3* 5' AACGCTTCACCGATGA 3', bases 1411 to 1426 in Fig. 2; HS106 in *aadB* 5' AGAGCGCAGATTTCG 3', complement of bases 1352 to 1367 in reference 1; HS57 in *aadB* 5' TCGACTGGCCTACAAA 3', bases 1722 to 1737 in reference 1; RH21 in *orfD* 5' CTTGTGTAGCAAGCGCG 3', complement of bases 2818 to 2834 in reference 38; and

HS120 in the 3'-conserved segment 5' GTAGGGCTTATTATGCA 3', complement of bases 147 to 166 in reference 37.

Nucleotide sequence accession number. Sequence data have been deposited with the EMBL and GenBank data libraries under accession number U13880.

RESULTS

Characterization of the multidrug resistance region of pBWH301. An *aacA* gene encoding an Ak^r determinant belonging to the antibiotic AAC(6')-I was previously isolated from pBWH301 on a 6.0-kb *Bam*HI fragment (19). This fragment also contained determinants for resistance to chloramphenicol and sulfonamides and a further aminoglycoside resistance determinant, *aadB*. The presence of the Su^r determinant and some characteristic restriction sites suggested that these genes were integron associated (19), and more-detailed mapping of the 6.0-kb *Bam*HI fragment (Fig. 1) revealed the patterns of sites characteristic of both the 5'- and 3'-conserved segments of *sull*-associated integrons (37). Thus, the cloned fragment extends from the *Bam*HI site near the outer end of the 5'-conserved segment to the first *Bam*HI site in the 3'-conserved segment.

By using deletion analysis, Hopkins et al. (19) localized the *aacA* gene, the Cm^r determinant, and the *aadB* gene to the region leftward of the second *Eco*RI site (Fig. 1) and determined their order as *aacA*-Cm^r gene-*aadB*. From the restriction map (Fig. 1), it can be estimated that approximately 1.2 kb of DNA with no assigned function lies between the *aadB* gene (identified by the characteristic restriction sites *Sac*I-*Xho*I-*Pvu*II-*Sph*I) and the 3'-conserved segment, indicating that one or more additional cassettes must be present. Sequences obtained with a primer within the *aadB* cassette and facing toward the 3'-conserved segment and a primer within the 3'-conserved segment and facing toward the insert region were identified as the 5' end of the *oxa2* cassette and the 3' end of the *orfD* cassette, respectively. These cassettes have previously been found in the integron In1 in the plasmid R46 (8, 13, 16, 38). The *oxa2* gene encodes a β -lactamase (OXA2) that confers resistance to a number of β -lactam antibiotics, including ampicillin and oxacillin, and *orfD* contains an open reading frame of unknown function. That the *oxa2* gene in pBWH301

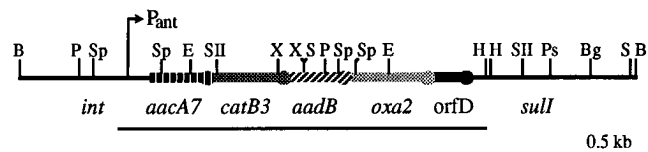


FIG. 1. Restriction map of the multidrug resistance region of pBWH301. Sequences assigned to the *sull*-associated integron 5'- and 3'-conserved segments are shown as thick black lines. Each gene cassette is indicated by a variation in pattern, and the cassette-associated 59-base elements are shown as patterned circles. A thin black line indicates the region sequenced in this study. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; P, *Pvu*II; Ps, *Pst*I; S, *Sac*I; SII, *Sac*II; Sp, *Sph*I; X, *Xho*I.

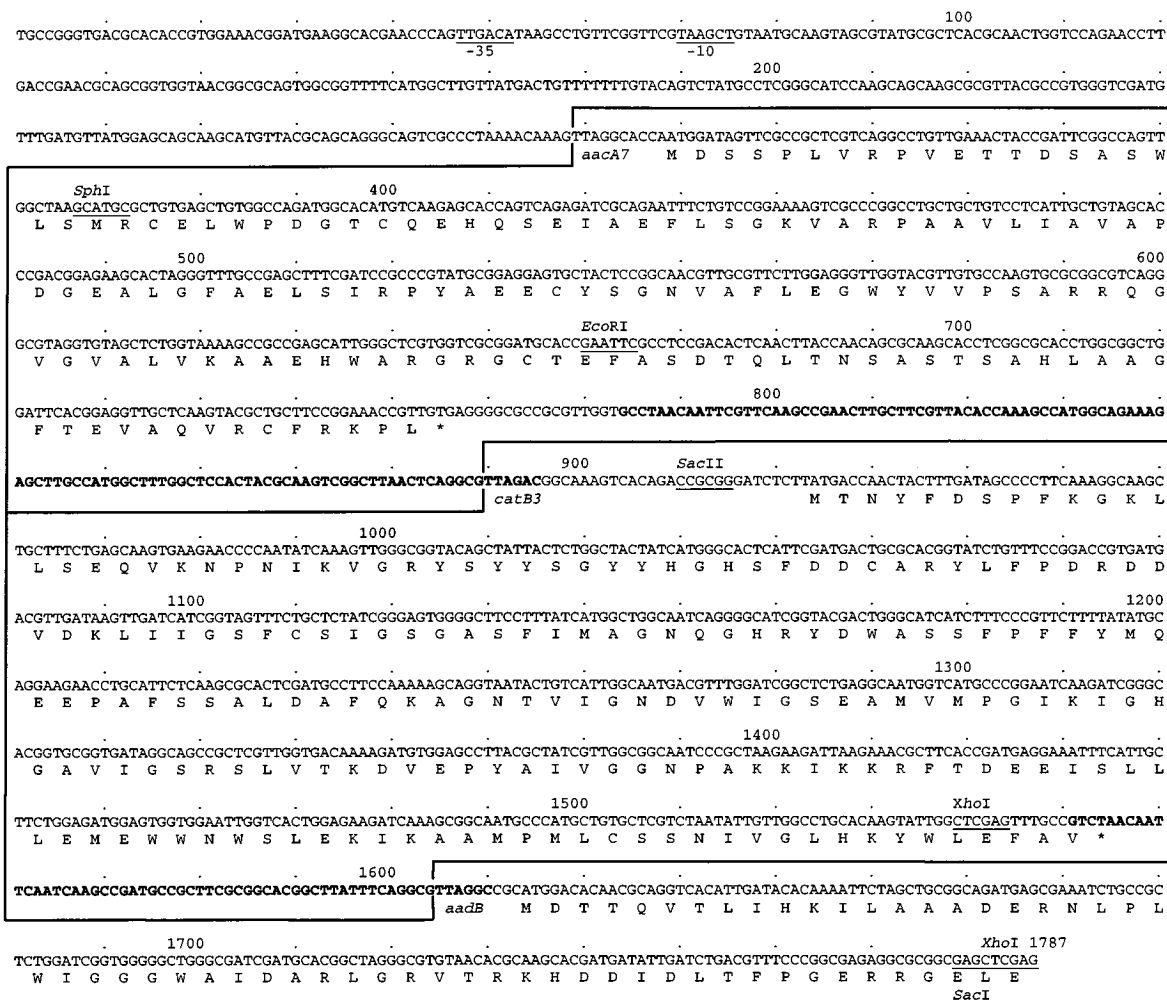


FIG. 2. Nucleotide sequence of the pBWH301 *aacA7* and *catB3* gene cassettes. The P_{ant} promoter -35 and -10 regions are underlined, the *aacA7* and *catB3* cassettes are boxed, and the 59-base elements are indicated in boldface type. The predicted amino acid sequences of AAC(6)-II and CATB3 are shown.

is active was confirmed by subcloning the 4.6-kb *Bam*HI-*Hin*dIII fragment (Fig. 1) into a vector that does not determine ampicillin resistance (see Materials and Methods). The resulting plasmid, pMAQ105, confers resistance to ampicillin with a 50% inhibitory concentration of 29 μ g/ml.

Sequence of the pBWH301 multidrug resistance region. The complete sequences of the inserted cassettes, together with part of the flanking 5'- and 3'-conserved segments, were determined (DNA data libraries accession number U13880). The sequences of the *aadB*, *oxa2*, and *orfD* cassettes and the 3'-conserved segment region were identical to previously reported sequences (*aadB* [1, 34], *oxa2* [8, 16, 38], *orfD* [13, 38], 3'-conserved segment [1, 37]). The sequence of part of the 5'-conserved segment and extending to the *Xho*I site in the *aadB* cassette is shown in Fig. 2. The sequenced region of the 5'-conserved segment differed at two positions from the corresponding region of In1 from R46 (16), and both differences are in the P_{ant} promoter. Three versions of P_{ant} have been identified (37) and have recently been classified according to their activity (6, 26). These are the weak promoter TGGACA N_{17} TAAGCT, the strong promoter TTGACA N_{17} TAAACT, and the hybrid promoter TGGACA N_{17} TAAACT, which is present in In1 (16) and has intermediate activity. The pBWH301 promoter TTGACA N_{17} TAAGCT (Fig. 2) repre-

sents a fourth version of P_{ant} that is also a hybrid of sequences found in the weak and strong variants of P_{ant} . This hybrid is also likely to have activity intermediate between that of the weak and strong P_{ant} promoters.

Generally, genes found inserted in integrons are part of a mobile gene cassette that consists of a single coding region followed by a 59-base element (4, 13, 14). In the region between the 5'-conserved segment and the beginning of the *aadB* cassette, open reading frames for the new aminoglycoside and Cm^r determinants were identified, and the predicted polypeptides are shown in Fig. 2. These genes have been designated *aacA7* and *catB3* (see below). The 59-base elements associated with these genes (boldface type in Fig. 2) were identified by searching downstream of the genes for inverted repeats that are closely related at their outer ends to the most recent consensus for 59-base elements (5). The *catB3* 59-base element is 60 bases long and is closely related to the earlier consensus for elements of this length (1, 13). The *aacA7* 59-base element is 110 bases long. Thus, both the *aacA7* and the *catB3* genes are part of typical cassette structures (boxed in Fig. 2), and the integron in pBWH301 contains a total of five integrated gene cassettes, *aacA7-catB3-aadB-oxa2-orfD*.

Aminoglycoside acetyltransferase resistance gene, *aacA7*. The promoter-proximal cassette contains an open reading


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AAC(6')-I1 MDSSPLVRPVETDTSASWLSMRCELWPDGTCQEHQSEIAEFLSGKVARPAAVLIIVAPDG
AAC(6')-Ic  MIVICDHDNLDAWLALRTALWPSGSPEDHRAEMREILAS--PHH-TAFMARGLDG
AAC(6')-Id  MIEACHSVECPGWLQRLRFLFWPQSDAHLAEMAIFVAE--PNRFAQFIAYDEAN
AAC(6')-If  MDEASLSMWVGLRSQWLWPDHSHYEDHILDSQHILSC--PKYVVSFLAINNQS
AAC(6')-Ig  MNIKPASEASLKDWLELRNKLWSD-SEASHLQEMHQLLAE---KYALQLLAYS-DH
AAC(6')-Ih  MNIMPISSEQLSDWLALRCLLWPDH-EDVHLQEMRQLITQ---AHRLQLLAYTDTQ
AAC(6')-Ij  MNIMPVSESLMADWLGLRKLKLLWPDH-DEAHLQEMRQLLQ---TQSLQLLAYSDTQ
. . . . . * . . * . . * . . . . . * . . . . . * . . . . . * . . . . . * . . . . . *
AAC(6')-I1 EALGFAELSIIRP-YAEECYSGNVAFLEGWVVPVPSARRQGVGVALVKAEEHWARGRGCTEF
AAC(6')-Ic  AFVAFAEVALRYDYVNGCESSPVAFLEGIYTAERARRQGWAARLIAQVQEWAKQQGCSEL
AAC(6')-Id  KPLGFVEAALRSYVNGTSSPVAFLEGGVYVLEPEARRRGIAHALVGAIVEIWARNRACTEF
AAC(6')-If  QAIADFADAARHDYVNGCESSPVVYLEGIFVPEQRGHGVAKLLVAVQDWGVAKGCTEM
AAC(6')-Ig  QAIAMLEASIRFEYVNGTETSPPVGFLEGIYVLPVHRRSGVATMLIRQAEVWAKQFSCTEF
AAC(6')-Ih  QAIAMLEASIRFEYVNGTQTSPPVAFLEGIYVLPVHRRSGIATGLVQQVEIWKQFCTEF
AAC(6')-Ij  QAIAMLEASIRFEYVNGTQTSPPVAFLEGIYVLPDYRRSGIATHLVQQVEAWAKPFQCTEF
. . . . . * . . * . . * . . . . . * . . . . . * . . . . . * . . . . . *
AAC(6')-I1 ASDTQLTNSASTSAHLAAGFTEVAQVRCFRKPL*
AAC(6')-Ic  ASDTDIANLDSQRLHAALDFAETERVVFYRKTGL*
AAC(6')-Id  ASDASTDNPESHRFHQSLGFKETERVVYFRKMLAPE*
AAC(6')-If  ASDAALDNHISYQMHQALGFETERVVFYRKRRIAG*
AAC(6')-Ig  ASDAALDNVISHAMHRSGLGFQETEKVVFYFVSKKID*
AAC(6')-Ih  ASDAALDNQISHAMHRSGLGFHETERVVFYFVKKKIG*
AAC(6')-Ij  ASDAALDNRISSHAMHRSGLGFHETERVVFYFVKKHIG*
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FIG. 3. Comparison of the derived amino acid sequences of the AAC(6')-I proteins AAC(6')-I1 (accession number U13880 [this study]), AAC(6')-Ic (accession number M94066 [36]), AAC(6')-If (accession number M55353 [36, 40]), the putative AAC(6')-Id protein (accession number X12618 [34, 36]), AAC(6')-Ig (accession number L09246 [25]), and AAC(6')-Ih and AAC(6')-Ij (accession numbers L29044 and L29045, respectively [24]). Alignments were derived with the Clustal V program (18). Identical amino acids are indicated with an asterisk; conservative amino acid substitutions are indicated with a dot. Substitutions were designated conservative if all amino acids fall within one of the following exchange groups: T, S, A, G, and P; R, K, and H; F, W, and Y; D, E, Q, and N; I, L, M, and V; and C (10).

frame of 456 nucleotides, extending from the ATG codon at positions 309 to 311 to a stop codon at positions 767 to 769 (Fig. 2). The predicted product of this open reading frame is 152 amino acids long (molecular weight, 16,358) and is related to several known AAC(6')-I proteins that form one of three distinct families of aminoglycoside acetyltransferases classified as AAC(6')-I (35). Because the predicted AAC(6')-I protein was between 31.6 and 39.1% identical and 50.7 and 57.1% similar to these proteins (Fig. 3) and confers the phenotypes characteristic of AAC(6')-I (19), it was designated AAC(6')-I1. The gene has been designated *aacA7* according to the standard nomenclature (29).

CAT gene, *catB3*. The second cassette, which determines resistance to chloramphenicol, contains an open reading frame of 627 bases that extends from the ATG codon at positions 924 to 926 to the stop codon at positions 1553 to 1555 and predicts a protein of 209 amino acids (molecular weight, 23,590) (Fig. 2). This protein is a member of a recently identified family of CATs, here designated CATB proteins, that are not related to the majority of previously identified CATs (31, 39). The new determinant, as the third identified member of this family, is designated CATB3 (*catB3*). The closest relative of CATB3 is CATB2, which is also cassette encoded and found in the integron in Tn2424 (31) (Fig. 4), and these two proteins share 82.3% identity and 91.3% similarity. CATB3 also shares 67.4% identity (79.4% similarity) with CATB1, which was found in a *Cm^r* isolate of *A. tumefaciens* and is not cassette determined (39), and 68.4% identity (84.4% similarity) with the product of an open reading frame (Ps orf) found in the chromosome of *P. aeruginosa* PAO1 (31).

In pairwise comparisons, the DNA sequences of the *catB1*, *catB2*, and *catB3* genes and the Ps open reading frame are closely related (61.1 to 73.8% identity) over the lengths of their coding regions (Fig. 5). However, there is essentially no similarity between the sequences flanking the genes. All four genes are related, except for a short region at their 3' ends, and 59%

of the nucleotides in the coding region are conserved in all four sequences. Over half of the sequence differences observed (Fig. 5) are changes in the third position of a codon, and many of these represent synonymous substitutions that are not reflected as differences in the amino acid sequences (Fig. 4). The *catB3* gene shares between 64.4 and 73.8% DNA sequence identity with each of the other open reading frames.

A further sequence, from an isolate of *Serratia* sp. 45 (41), was found to be between 64.9 and 84.9% identical to the first half of the *catB1*, *catB2*, *catB3*, and Ps open reading frame coding regions (Fig. 5). Examination of the complete sequence of Toriya et al. (41) revealed that this region is located between the integron 5'-conserved segment and an *aacA4* gene and appears to represent the 5' half of an integron-associated *catB* cassette that has become fused to an *aacA4* gene cassette (see Discussion). A polypeptide predicted from an open reading frame contained in this region is also closely related to the N-terminal half of the CATB proteins (Fig. 4), and the partial gene was designated *catB4Δ1*.

Integrase-dependent mobilization of the gene cassettes.

While gene cassettes can be identified by the presence of a gene coding sequence and an associated 59-base element, they are ultimately defined by their ability to be independently mobilized by integrase-mediated site-specific recombination (4). To confirm that the new cassettes identified here are mobile, Int-mediated excision of cassettes from pMAQ105 was studied. High levels of Int were provided by introducing the plasmid pSU2056, and plasmids that had lost one or more cassettes were identified by screening for the loss of antibiotic resistance as described previously (4). The order of cassettes in these plasmids was determined by restriction mapping, and some of the configurations identified are shown in Table 2. The fact that plasmids that have lost only the *aacA7* cassette (pMAQ137) or have retained only the *aacA7* cassette (pMAQ135) were obtained confirms that this cassette is a discrete unit. Plasmids retaining only the *catB3* cassette

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CATB3      MTNYFDSPFKGKLLSEQVKNPNIKVGRYSYYSYGYHGHSFDDCARYLFPDRDDVDKLI I
CATB2      MTNYFESPFKGLLSEQVKNPNIKVGRYSYYSYGYHGHSFDDCARYLLPDRDDVDQLI I
CATB4Δ1    MKNYFNPFKGLLSEQVKNPNIRVGRYSYYSYGYHGHSFDEFCARYLFPDRDDVDKLI I
CATB1      MENYFESPFRTLDKQVKSPLVVGKYSYYSYGYHGHSFEDCARYLLPD-EGADRLVI
Ps orf     MGNFYFESPFKGLLSEQVSNPNIRVGRYSYYSYGYHGHSFDDCARYLMPDRDDVDKLI I
          * * * * * . * * * * * . * * * * * . * * * * * . * * * * * . * * * * *
          * * * * * . * * * * * . * * * * * . * * * * * . * * * * * . * * * * *

CATB3      GSFCISIGSGASFIMAGNQGHRYDWASSFFPFYMQEPAFSSALDAFQKAGNTVIGNDV
CATB2      GSFCISIGSGARFIMAGNQGHRYDWSSFFPFYMNPEPAFAKSVDAFORAGDTVIGSDV
CATB4Δ1    GSFCISIGSGASFIMAGNQGHRHDWASSFFPFYMQEEPAS*
CATB1      GSFCISIGSAAFIMAGNQGHRNEWISTFPPFFMPEVPEFENAANGYLPAGDTVIGNDV
Ps orf     GSFCISIGSAAFIMAGNQGHRAEWASTFPPFFMHHEEPVFAVAVNGYQAGDTLIGHDV
          ***** * * * * * . * * * * * . * * * * * . * * * * * . * * * * *

CATB3      WIGSEAMVMPGIKIGHGAVIGSRSLVTKDVEPYAIVGGNPAKKIKRFTDEEISLLE
CATB2      WIGSEAMIMPGIKIGHGAVIGSRALVAKDVEPYTIVGGNPAKSIRKRFSEEEISMLLD
CATB1      WIGSEAIMPGITVGDGAVIGTRALVTKDVEPYAIVGGNPAKTIRKRFDDDSIALLE
Ps orf     WIGTEAMFMPGVRGVHGAIGSRALVTGDVEPYAIVGGNPARTIRKRFSDGDIQNLLE
          ***** * * * * * . * * * * * . * * * * * . * * * * * . * * * * *

CATB3      MEWWNWSLEKIKAMPMLCSSNIVGLHKYWFLEFAV*
CATB2      MAWWDWPLEQIKEAMPFLCSSGIASLYRRWQGTSA*
CATB1      MKWGWPAERLKAAMPLMTSGNVAALYRFWRSDSL*
Ps orf     MAWWDWPLADIEAAMPPLLCTGDIPALYRHWKQRQATA*
          * * * * * . * * * * * . * * * * * . * * * * * . * * * * *

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FIG. 4. Comparison of the derived amino acid sequences of the CATB proteins and of a *P. aeruginosa* open reading frame. These proteins are designated as follows: CATB1 from *A. tumefaciens* (accession number M58472 [39]); CATB2 from the integron in Tn2424 (accession number M80188 [31]); CATB3 (accession number U13880 [this study]); and CATB4Δ1, identified in this study (see Discussion) in a *Serratia* sp. 45 isolate (accession number S49888 [41]). Ps orf is the predicted protein from a *P. aeruginosa* PAO1 open reading frame (31). See the legend to Fig. 3 for an explanation of the symbols.

(pMAQ126) were also identified. Similarly, the *aadB*, *oxa2*, and *orfD* cassettes are also individual mobile units. The sequences of the boundaries in each excision product shown in Table 2 were determined (data not shown) and confirmed that the cassettes behaved as discrete mobile units that were precisely excised or rearranged by Int, as described previously (4, 5).

DISCUSSION

The *aacA7* gene characterized in this study was predominantly responsible for the Ak^r outbreak at Hospital Vargas in Venezuela, because 2 of 21 isolates examined by Hopkins et al. (19) contained plasmids equivalent to pBWH301 (sequenced here) and a further 12 isolates were equivalent to pBWH300, which from the published restriction map appears to contain an integron with a single integrated *aacA7* gene cassette. That this is so was confirmed by sequencing the boundaries of the cassette in pBWH300 (data not shown). The remaining isolates were not examined in this study. The AAC(6')-II protein determined by *aacA7* is related to several other AAC(6')-I proteins (Fig. 3). Only one other member of this family [AAC(6')-Id] is encoded in a gene cassette, found in the plasmid pBP201 (34). The open reading frame found in this cassette was previously designated *orfB* (13), but the *orfB* product has since been shown to be similar to other AAC(6') proteins (36, 40) and has been designated AAC(6')-Id (36). However, the two cassette-determined AAC(6')-I proteins are no more related to one another than to other members of the family (Fig. 3), suggesting that these two cassettes have independent origins.

A Cm^r determinant was also present in pBWH301 (19). This new gene, *catB3*, encodes a CAT that belongs to a recently identified family of CAT proteins (31, 39), here designated the CATB family. Both the *aacA7* and *catB3* genes are contained in mobile gene cassettes which are integrated in an integron with typical 5'- and 3'-conserved segments present in pBWH301. The pBWH301 integron contains five integrated

gene cassettes in the order *aacA7-catB3-aadB-oxa2-orfD*, and all of the cassettes were mobilized by the integron-encoded DNA integrase.

While gene cassettes generally include a single gene and a 59-base element, deletion events with end points in two adjacent cassettes can potentially lead to the fusion of the two cassettes to form a new cassette. Fused cassettes should remain mobile because they contain a core site from the 5' end of one cassette and a 59-base element from the 3' end of the second cassette. A case in which this is likely to have occurred was identified in this study. An incomplete *catB* gene cassette was found in the published sequence of a region containing an *aacA4* gene isolated from an Ak^r *Serratia* sp. 45 isolate (41). Though the authors did not note this, the sequence consists of the last 253 bases of an integron 5'-conserved segment (bases -578 to -326 in reference 41) separated by 325 bases (bases -325 to -1) from an *aacA4* gene (bases 1 to 555). The complete *aacA4* gene cassette is 637 or 638 bp long and has been shown to be mobile (4). However, the first 22 bases of the *aacA4* cassette are not present in the sequence reported by Toriya et al. (41). In this study, part of the region between the 5'-conserved segment and the *aacA4* coding region was found to be between 64.9 and 84.9% identical to the first half of the *catB1*, *catB2*, and *catB3* genes and of the Ps open reading frame. An open reading frame (bases -294 to +4 in reference 41) with a predicted product closely related to the N-terminal half of the CATB proteins (Fig. 4) was identified in this region, and we conclude that this open reading frame represents a truncated *catB* gene designated *catB4Δ1*. We therefore propose that the region (-325 to -3) between the identifiable 5'-conserved segment and *aacA4* cassette sequences was originally part of a cassette that contained the complete *catB4* gene. Both the 3' half of the *catB4* gene cassette and the 5' non-coding region of the *aacA4* cassette appear to have been deleted to produce a single fused cassette that includes the *catB4Δ1* gene and the complete *aacA4* gene, as is illustrated in Fig. 6a. Whether the 3' end of the *aacA4* cassette is present is

```

catB3                               TTAGACGGCAAAGTCACAGACCGGGATCTCTT
catB2                               TTAGGCGACGCGTGGAGTCGCTCTAGAATTTTCGGGTACAATTTT
catB4Δ1                             TTAGACGGCAAAGAAAGGTTCCACGAACCTCTG
catB1   TCGGTTTTCGCTTGGCGTATGCTTGCCTGAAGGCAAGCTAAATGCGAACAGATGCAGTGAGATTATTTCA
Ps orf  TCACGCCAGGCTGGCCCGCTATCCCGCAGGCGCTACCCCTTGGCGGTTTCGACGGACAGGGACGACGGT
      **      *      **      *

catB3   ATGACCAACTACTTTGATAGCCCCTTCAAAGGCAAGCTGCTTTCTGAGCAAGTGAAGAACCCTAATATCA
catB2   ATGACGAATTATTTTGTAGAGTCCCTTCAAAGGGAAGCTTCTGACTGAGCAGGTGAAGATCCGAACATCA
catB4Δ1 ATGAAAAACTACTTTAACAGCCCTTCAAAGGGAACTTCTTTCTGAGCAAGTGAAGATCCAAATATCA
catB1   ATGGAAAACATTTTCGAAAGCCCATTCGGGGGCATTAAGTACGACAGCAGGTGAAGAGCCCGAACCTGG
Ps orf  ATGGGCAACTATTTTCGAGAGCCCTTCAAGGGCAAGCTGCTCTCGGAACAGGTGAGCAACCCAAACATTC
      ***  * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

catB3   AAGTTGGGCGGTACAGCTATTACTCTGGCTACTATCATGGGCATCTATTCGATGACTGCGCACGGTATCT
catB2   AGGTAGGGCGGTATAGCTACTATTCCGGCTATTACCATGGGCATCTGTTGATGATTGTGCTCGTACCT
catB4Δ1 GAGTAGGCCGGTATAGCTATTACTCTGGCTACTATCACGGGCATCTATTCGATGAATGCGCGCATACTT
catB1   TGGTCGAAAATACAGCTATTATTCGGCTATTACCAGGCCACAGTTTCGAGGATTGGGCCCGGTACCT
Ps orf  GGGTGGGGCGCTACAGCTACTACTCCGGCTACTATCACGGGCATTCCTTCGACGACTGCGCCCGTACCT
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

catB3   GTTTCGGACCGTGATGACGTTGATAAGTTGATCATCGGTAGTTTCTGCTCTATCGGGAGTGGGGCTTCC
catB2   TCTACCAGACCGTGATGACGTTGATCAGCTGATTATCGGCAGCTTCTGCTCCATCGGATCAGGCGCACGT
catB4Δ1 GTTTCAGATCGTGATGACGTTGATAAATGATCATTTGGCAGCTTTTGTCTATAGGAAGCGGGGCTTCC
catB1   GGTGCCCGA---TGAGGGCGCGGACAGGCTGGTGATCGGAAGCTTCTGCTCGATCGGTTCCGGCCCGCC
Ps orf  GATGCCGACCGCGACGCTGGACAAGCTGGTATCGGCAGTTTCTGTTGATCGGCACTGGCCCGCC
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

catB3   TTTATCATGGCTGGCAATCAGGGGCATCGGTACGACTGGGCATCATCTTCCCCTTCTTTATATGCAGG
catB2   TTTATATATGGCTGGGAATCAAGGCCACCGATATGATTGGGTCTCTTCTTCCCCTTCTTCTACATGAACG
catB4Δ1 TTCATCATGGCTGGCAATCAGGGGCATCGGCATGACTGGGCATCATCTTCCCCTTCTTCTATATGCAAG
catB1   TTCATCATGGCGGCAATCAGGGGCACCGCAACGAATGGATCAGTACCTTCCCCTTCTTCTCATGCCGG
Ps orf  TTCATCATGGCCGCAACCCAGGGACACCGCCGAATGGGCATCGACCTTCCCCTTCCACTTCATGCACG
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

catB3   AAGAACCTGCATTCTCAAGCGCCTCGATGCCTTCCAAAAGCAGGTAATCTGTCATTGGCAATGACGT
catB2   AGGAGCCCGCTTTCGAAAATCAGTTCGATGCATTCACGCGGCTGGCGACACAGTTATAGGAAGTGATGT
catB4Δ1 AGGAGCCTGCT
catB1   AGGTGCCGAATTCGAGAATCCGCCAACGGTTATCTGCCGGCGGGGACACCGTCATCGGCAACGATGT
Ps orf  AAGAGCCGCTTCGCCGCGCTGAACGGTATCAGCCAGCCGGCGACACCGTGCATCGGCGATGAAGT
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

catB3   TTGGATCGGCTCTGAGGCAATGGTTCATGCCCGAATCAAGATCGGGCAGGTGCGGTGATAGGCAGCCGC
catB2   GTPGATCGGTTTCGGAGCCATGATCATGCCCGGATCAAGATCGGGCATGGAGCGGTGATAGGTAGCCGC
catB1   CTGGATCGGTTTCGGAAGCGATCATCATGCCCGCATCACCGTTCGGTTCGATGGCGCGGTGATCGGAACCGCG
Ps orf  CTGGATCGGTTACCGAGCGATGTTTCATGCCCGGCTACGGGTTCGGGCACCGGACCATCATCGGCGCCGC
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

catB3   TCGTTGGTGACAAAAGATGTGGAGCCTTACGCTATCGTTGGCGCAATCCCGCTAAGAAGATTAAGAAAC
catB2   GCTTTGGTTGCCAAGACGTTGAACCTTACACCATAGTGGGGGAAACCCCTGCAAAGTGCATTAGGAAGC
catB1   GCGCTGGTGACGAAGGACGTCGAGCCCTACGCCATCGTTCGGCGGCAACCCCGCAAAAACGATCCGCAAGC
Ps orf  GCGCTGGTGACCGCGATGTCGAGCCCTATGCCATCGTTCGGCGGTAACCCCGGACCATTCGTAAGC
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

catB3   GCTTACCAGTGAAGAAATTTTCATGCTTCTGGAGATGGAGTGGTGAATTTGGTCACTGGAGAAGATCAA
catB2   GCTTTTCTGAAGAAGAAATTTCTATGCTTTTAGATATGGCTTGGTGGGATTGGCCGCTGGAACAAATCAA
catB1   GGTTTGACGACGATAGCATCGCCCTGCTGCTCGAGATGAAATGGTGGGGCTGGCCGCGGAGCGGCTGAA
Ps orf  GCTTTTCCGATGCGGATATCCAGAACCCTGCTGGAATGGCCTGGTGGGACTGGCCACTGGCCGATATCGA
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

catB3   AGCGGCAATGCCATGCTGTGCTCGTCTAATATGTTGGCCTGCACAAGTATGGCTCGAGTTTGGCCGTC
catB2   GGAAGCAATGCCPTTTCTTTGTTTCGCTTGGCATTGCCAGCCTGTATCGTTCGCTGGCAAGGCACAAGCGCC
catB1   AGCTGCAATGCCGCTGATGACAGCGGCAATTCGCGGCGCTCTATCGTTTCTGGCGGTCCGATAGCCTC
Ps orf  GGCAGCCATGCCACTGCTGTGACTGGGGATATCCCGCCTTGTACCAGCACTGGAAACAGCGCCAGGCC
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

catB3   TAACAATTCATCAAGCCGATGCCGCTTCGCGGCACGGCTTATTTTCAGGCG
catB2   TAACAATACGCTACACACGGACAAATTACCCCTTGCCTCCTAATTTGCCGGTGAGCGTGGCG
catB1   TAGCGGTAAGATTGCCGGCCGCTCCAGACAGAGCGGGCGGCATTTCGCTGGACAGGACAGATGATGT
Ps orf  TCGGCCTGACGGTTGGACGAAGGAGTTCGAG
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
    
```

FIG. 5. Comparison of sequences of the *catB* genes and a *P. aeruginosa* open reading frame (Ps orf). For cassette-associated genes only the sequence assigned to each cassette is shown, and the 59-base elements are underlined. The start and stop codons are in boldface. Asterisks indicate identical bases in all four sequences. The sequences shown are those for *catB1* (accession number M58472 [39]), *catB2* (accession number M80188 [31]), *catB3* (accession number U13880 [this study]), and *catB4Δ1* (accession number S49888 [41]). All accession numbers refer to the EMBL and GenBank databases. The Ps orf sequence is from *P. aeruginosa* PA103, bases 1 to 261 are bases 803 to 1163 in reference 42 (accession number X52462), and bases 262 to 731 are unpublished (23). The PAO1 sequence used to derive the Ps orf amino acid sequence shown in Fig. 4 differs at only eight positions, leading to three amino acid substitutions (23).

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TABLE 2. Products of Int-mediated excision of cassettes from pMAQ105

Plasmid ^a	Antibiotic resistance		Cassette order
	Selected	Retained ^b	
pMAQ135	Ak ^r	Ak ^r	<i>aacA7</i>
pMAQ126	Cm ^r	Cm ^r	<i>catB3</i>
pMAQ134	Gm ^r	Gm ^r	<i>aadB</i>
pMAQ137	Cm ^r	Ap ^r Cm ^r Gm ^r	<i>catB3-aadB-oxa2-orfD</i>
pMAQ138	Gm ^r	Ap ^r Gm ^r	<i>aadB-oxa2</i>
pMAQ139	Cm ^r	Ak ^r Cm ^r Gm ^r	<i>aacA7-catB3-aadB</i>
pMAQ140	Ak ^r	Ak ^r Ap ^r	<i>aacA7-oxa2</i>
pMAQ141	Cm ^r	Ap ^r Cm ^r	<i>catB3-oxa2-orfD</i>
pMAQ146	Ak ^r	Ak ^r Ap ^r Gm ^r	<i>aacA7-aadB-oxa2</i>
pMAQ127	Cm ^r	Ak ^r Cm ^r	<i>aacA7-catB3-orfD</i>
pMAQ133	Cm ^r	Ak ^r Ap ^r Cm ^r	<i>aacA7-catB3-oxa2</i>

^a Plasmids are derivatives of pMAQ105 that have lost one or more complete cassettes as a result of Int-mediated site-specific recombination.

^b Screened for resistance to amikacin (5 µg/ml), ampicillin (20 µg/ml), chloramphenicol (10 µg/ml), and gentamicin (5 µg/ml).

not known, because the cassette-associated sequences extend beyond the end of the reported sequence.

A cassette fusion may also explain the relationships between the orfD cassette found integrated in the pBWH301 (this study) and R46 (13, 38) integrons and the closely related orfF cassette found in the integron in pEH1 (17) and the orfE cassette found in In4 in TnI696 (13, 43). The orfD and orfF cassettes are 88.8% identical over their full 320-bp length. The orfE cassette is 58 bp shorter, but the last 197 bp, including the 59-base element, share 72.1 and 75.1% identity with the corresponding regions of the orfF and orfD cassettes, respectively (Fig. 6b). The 5' end of the orfE cassette (bases 1 to 65) is not related to any other region of the orfD and orfF cassettes, indicating that the orfE cassette did not arise by a simple deletion internal to a cassette related to orfD and orfF. We propose that either the orfE cassette or the orfD and orfF cassettes were created by the fusion of two gene cassettes, but

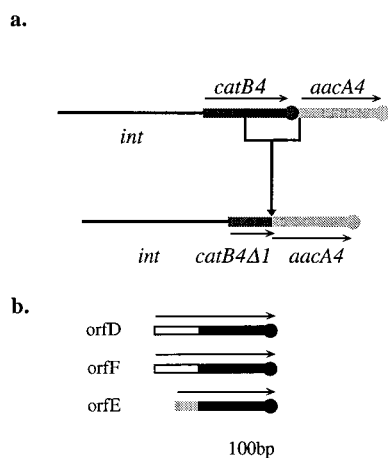


FIG. 6. A model for cassette fusion. (a) Fusion of the putative *catB4* gene cassette with the *aacA4* gene cassette to produce the configuration found in a *Serratia* sp. 45 isolate. Thin black lines represent the 5'-conserved segment. (b) Schematic comparison of the orfD, orfF, and orfE, gene cassettes. The black region of each cassette represents the last 197 bp, which are between 72.1 and 88.8% identical. The white regions at the 5' end of the orfD and orfF cassettes also share 78.0% identity. The unrelated 5' end of the orfE cassette is shown as a shaded rectangle. In both panels a and b, filled circles represent the 59-base elements and arrows indicate the open reading frames.

because no function has yet been identified for any of these open reading frames, it is not possible to conclude which cassette represents the fusion product. However, both the orfD and the orfE cassettes have been demonstrated to be discrete Int-mobilizable units (references 4 and 5 and this study).

The origins of transmissible antibiotic resistance determinants have long been the subject of speculation. Two hypotheses have been put forward: namely, that resistance genes originated in antibiotic-producing organisms such as the actinomycetes or that they have arisen from housekeeping genes from various microorganisms (see reference 9 for review). Although evidence to support both of these proposals has been found in comparisons of the sequences of plasmid- or transposon-determined resistance proteins with those from streptomycetes or with housekeeping genes, only recently has any case been identified in which this relationship is so close that the DNA sequences are significantly related (7, 11, 20). The close relationship of the transmissible, cassette-associated *catB* genes to a chromosomal open reading frame found in *P. aeruginosa* is another case in which similarity at the DNA level is detectable. The coding regions of the *catB* genes and the *P. aeruginosa* open reading frame share a high level of identity (61.1 to 73.8%), but the similarity does not extend beyond the start and stop codons (Fig. 5). The close relationship of these genes suggests that they have evolved either from one another or from a common ancestral gene. Indeed, it is possible that the Ps open reading frame or the equivalent open reading frame from a related species is the ancestor of the cassette-associated *catB* genes. It will be of interest to determine if this open reading frame is present in all pseudomonads. No function has yet been assigned to the *P. aeruginosa* open reading frame, although it is possible that it contributes to the intrinsic resistance of *Pseudomonas* spp. to chloramphenicol. In *P. aeruginosa* PA103, the open reading frame overlaps the *regB* gene, which is implicated in the expression of exotoxin A (42), and CAT activity was not detectable in this strain (23). In PAO1, the *regB* initiation codon is not present (42) and low levels of CAT activity have been detected (23). However, it is not known if the CAT activity in this strain is due to the fact that *regB* is not translated or to point mutations within the gene or other changes that potentially affect expression of the open reading frame (23). The location (plasmid or chromosomal) of the *catB1* gene in *A. tumefaciens* has not been determined, and it will also be of interest to determine if this gene is acquired or is present in the chromosome and only expressed in some strains.

The existence of several distinct but closely related genes within cassettes (e.g., *catB2*, *catB3*, and *catB4Δ1*) can potentially be explained in two ways. One possibility is that a single ancestral gene became part of a mobile gene cassette, presumably by acquiring a 59-base element, and that this cassette has subsequently evolved to give rise to the distinct *catB2*, *catB3*, and *catB4* cassettes. Alternatively, several closely related genes may have been independently converted to mobile cassettes. Because the two complete *catB* gene cassettes, *catB2* and *catB3*, are related only between the start and stop codons of the genes and have very different 59-base elements (Fig. 5), it is tempting to speculate that these cassettes represent the products of two separate cassette creation events. However, the mechanism by which these cassette genes acquire 59-base elements is unlikely to be elucidated until the origin of the cassette-associated genes is determined.

ACKNOWLEDGMENTS

We thank John D. Hopkins for supplying the plasmids pJH213 and pJH221 and Susan A. Kaye and Barbara H. Iglewski for kindly sup-

plying the DNA sequence of the *P. aeruginosa* PA103 open reading frame prior to publication.

This work was supported by a grant from the Australian Research Council.

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