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Aminoglycoside Acetyltransferase 3-IV (*aacC4*) and Hygromycin B 4-I Phosphotransferase (*hphB*) in Bacteria Isolated from Human and Animal Sources

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Members of the family *Enterobacteriaceae* harboring an enzyme of the aminoglycoside acetyltransferase 3 class (AAC-3-IV) (apramycin and gentamicin resistance) and hygromycin B phosphotransferase 4 (HPH-4-I) (hygromycin B resistance) have been isolated from human clinical sources in Europe. A cluster of genes containing IS*140*, *aacC4*, and *hphB* was found in these strains. We demonstrate by Southern hybridization that this cluster is identical to the operon found in animals that also contains insertion sequences belonging to the IS*6* family. This provides another example of presumptive transfer of antibiotic resistance genes between bacteria of animal and human origin.

Apramycin and hygromycin B are two members of the aminoglycoside family of antibiotics which have been used extensively for veterinary applications but have never been employed in human therapy. Gram-negative bacterial strains resistant to these antibiotics have been isolated from several different animal sources, and the associated resistance mechanisms have been characterized (1, 4, 9, 13). Apramycin resistance is due to N acetylation by an enzyme of the aminoglycoside acetyltransferase 3 class (AAC-3-IV), and hygromycin inactivation is due to O phosphorylation by hygromycin B phosphotransferase 4 (HPH-4-I). The corresponding genes (*aacC4* and *hphB*) have been cloned and sequenced (1, 7, 10); *aacC4* and *hphB* form part of an antibiotic resistance gene operon, which is transcribed from a promoter 5′ to the *aacC4* gene (1, 10).

AAC-3-IV, but not HPH-4-I, is capable of modifying a number of other aminoglycoside antibiotics, including gentamicin and tobramycin. Since the latter are used widely in human therapy and since bacterial resistance due to several different AAC-3 types has been generally reported, it might be anticipated that AAC-3-IV would be found in aminoglycoside-resistant isolates from humans. This has been noted recently in Europe (R. Gomez-Lus, J. Gil, M. L. Gomez-Lus, J. Castillo, E. Bouza, and M. C. Rubio, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 400, 1987; E. Chaslus-Dancla, Y. Glupczynski, G. Gerbaud, M. Lagorce, J.-P. Lafont, and P. Courvalin, Program Abstr. Réunion Interdisciplinaire de Chimiothérapie Antiinfectieuse Paris, abstr. no. 251/P13, 1988; Y. Glupczynski, J. Dewit, R. S. Hare, G. H. Miller, and E. Yourassowsky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, A-82, p. 14) and in the United States (R. S. Hare, K. J. Shaw, F. J. Sabatelli, L. Naples, S. Kocsi, A. Cacciapuoti, F. Menzel, C. Cramer, and G. H. Miller, 29th ICAAC, abstr. no. 675, 1989). It was of interest to see whether the AAC-3-IV of such isolates was encoded by the same *aacC4* as in isolates from animals, and more pertinently to determine whether or not the apramycin resistance gene in the human clinical isolates was associated with *hphB* in the same configuration as in veterinary isolates. We demonstrate by restriction analysis and Southern hybridization that this was the case, consistent with the notion that resistance gene transfer has taken place between bacterial populations in animals and humans.

MATERIALS AND METHODS

Strains. Plasmids pIP1508, pIP1509, and pIP1510 (Table 1) were described previously (2), had been isolated from *Salmonella typhimurium* or *Escherichia coli* in calf feces in France, and were a gift from E. Chaslus-Dancla (Institut National de la Recherche Agronomique, Tours, France). Plasmids pUZ7852, pUZ7874, pUZ6776, pUZ6734, and pUZ7343 were isolated from *E. coli*, *Klebsiella pneumoniae*, or *Serratia marcescens* in the blood or urine of patients in Madrid or Zaragoza, Spain. *E. coli* 86061324 of human origin was obtained from G. Miller (Schering-Plough). The plasmids from these strains encoded resistance for several aminoglycoside antibiotics (Table 2) and other antimicrobial agents (results not shown). The calves had been intensively treated with apramycin and gentamicin; some of the human patients (from whom *K. pneumoniae* 34 and *E. coli* 6734 were isolated) had been treated with gentamicin. Hygromycin B was not known to have been used in any instance.

The *E. coli* strain harboring plasmid pWP7b (see Fig. 3A) used for preparing the IS*140* and *aacC4* gene probes used for hybridization has been previously described (1). Plasmid pPM732 (unpublished data), a gift of P. Mazodier, is a multicopy derivative of pLG61 (7) containing the *hphB* gene from pJR225 (4).

Microbiological techniques. Enterobacterial strains were isolated on Drigalski or MacConkey medium, and an API system (API-System, La Balme les Grottes, France) was used to identify the strains. Antibiotic resistance was determined by the disk diffusion test on Mueller-Hinton agar. MICs were determined on the same medium (5).

Conjugation. *S. typhimurium*, *E. coli*, *S. marcescens*, and *K. pneumoniae* strains were conjugated overnight (15) in

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TABLE 1. Designations and origins of strains and plasmids

Donor strain	Yr of isolation	Origin	Recipient strain	Transconjugant	Plasmid designation
Salmonella sp.	1977	Horse	C600		pWP7b
S. typhimurium BN8700	1984	Calf feces	BM14	BM8701	pIP1508
S. typhimurium BN8900	1984	Calf feces	BM14	BM8906	pIP1509
E. coli BN4100	1984	Calf feces	BM21	BM4101	pIP1510
E. coli 7852	1988	Human urine	J62-1	E/7852	pUZ7852[a]
E. coli 7874	1988	Human urine	J62-1	E/7874	pUZ7874
K. pneumoniae 34	1986	Human blood	J62-1	E/6776	pUZ6776
E. coli 6734	1988	Human blood and urine	J62-1	E/6734	pUZ6734
S. marcescens 343	1988	Human urine	J53-2	E/7343	pUZ7343
E. coli 86061324	1986	Human	ND[b]	ND	Several plasmids

[a] Transconjugant E/7852 probably harbors several plasmids, as seen in the profile in Fig. 1.
[b] ND, Not done.

liquid brain heart infusion medium with recipient strain *E. coli* K-12 BM14 (*azi met pro*), BM21 (*gyrA*) (2), J62-1 (F^- *his lac nalA pro trp*), or J53-2 (*met pro rif*), respectively.

Assay for aminoglycoside-modifying enzymes. Crude bacterial extracts of recipient strains were prepared by sonication, and the enzymes were assayed by a phosphocellulose paper-binding assay (8).

DNA preparation and agarose gel electrophoresis. Plasmid DNA was prepared by alkaline lysis (11), except that the volume of culture used for the minipreparation was increased from 1.5 to 20 ml. Agarose gel electrophoresis was performed with 0.7% agarose in TAE buffer (11). It was found that the presence of 0.2% sodium dodecyl sulfate in the bromophenol blue dye buffer enhanced the penetration of undigested plasmid DNA in the agarose. The physiochemical basis of this phenomenon is not known.

Probe preparation. The probes used for Southern hybridization were intragenic fragments obtained from plasmid pPM732 (probe *hphB*) or pWP7b by digestion and cloning in the multicopy vector pUC18 or pUC19 (16) in order to obtain recombinant plasmid pDSA701 (probe IS*140*) or pDSA702 (probe *aacC4*). The probe for IS*140* consisted of the 0.2-kilobase (kb) *Pst*I-*Sal*I fragment of the IS*140* element, the probe for *aacC4* was a *Sac*I 0.75-kb intragenic fragment, and the probe for *hphB* was an *Eco*RI-*Sac*II 0.55-kb intragenic fragment. After digestion of pPM732 or the recombinant plasmids, these fragments were then isolated by electrophoresis on 8% acrylamide thin gels and subsequent elution by passive diffusion in 0.3 M sodium phosphate–0.1% sodium dodecyl sulfate buffer at 42°C.

Southern hybridization. For Southern hybridization (14) after electrophoresis, the digested DNA of plasmids was transferred from 0.7% agarose to a Hybond N filter (Amersham International plc, Little Chalfont, England) in 10× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The DNA was then covalently bonded to the filter by 5 min of irradiation with UV light at 312 nm and prehybridized for 3 h on an oscillator at 42°C in a solution containing 50% formamide, 5× SSC, 1× Denhardt solution, and 0.2 mg of herring testis DNA (denatured by microwaves or boiling) per ml. The probes (about 50 ng of DNA) were labeled by nick translation with 0.75 MBq (20 μCi) of deoxycytidine-5′-[α-^{32}P]triphosphate at 110 TBq/mmol (Amersham 5000 kit). The labeled probes were purified by chromatography on Sephadex G-50 (NICK column; Pharmacia, Uppsala, Sweden). After denaturation by boiling and cooling on ice, the probe was added to the prehybridization solution and hybridization was performed overnight at 42°C. The filter was washed twice in 1× SSC buffer with 0.1% sodium dodecyl sulfate at 50°C for 20 min. Autoradiography was done for 1 to 4 h without an amplifying screen. Stripping was performed at 45°C in a 0.4 M NaOH solution for 20 minutes, followed by neutralization in 0.1× SSC–0.1% sodium dodecyl sulfate–0.2 M Tris hydrochloride, pH 7.5.

Chemicals. Antibiotics were obtained as follows: gentamicin C1a, Schering Corp., Bloomfield, N.J.; apramycin and hygromycin B, Eli Lilly & Co., Indianapolis, Ind.; and amikacin, Bristol Laboratories, Syracuse, N.Y. Restriction enzymes were obtained from Pharmacia (*Bgl*II) and Appligene (*Sal*I and *Pst*I). Radiochemicals were obtained from Amersham.

TABLE 2. MICs of various antibiotics against the nine strains used for hybridization

Strain	MIC (μg/ml)			
	Amikacin	Gentamicin C1a	Apramycin	Hygromycin B
BM14	1	1	4	32
BN8701	1	16	1,024	256
BN8906	1	16	1,024	256
BN4101	1	16	1,024	256
E/7852	2	32	>1,024	512
E/7874	2	32	>1,024	1,024
E/6776	2	64	>1,024	1,024
E/6734	4	64	>1,024	512
E/7343	8	64	>1,024	512
86061324	1	32	>1,024	256

RESULTS

Resistance to hygromycin B. All the apramycin- and gentamicin-resistant strains, regardless of source, were also resistant to hygromycin B (Table 2). Resistance enzyme analyses showed that these strains harbor both *aacC4* and *hphB*, according to their typical substrate profiles (reference 2 and results not shown). Table 3 shows the results for gentamicin C1a, apramycin, hygromycin B, and amikacin. Apramycin was modified only by acetylation and hygromycin B was modified only by phosphorylation, as previously reported (4, 10).

Resistance plasmids. DNA was isolated from each strain after conjugation to *E. coli* (2), and the result of electrophoretic separation of fragments from the different plasmids after digestion with *Sal*I and *Bgl*II is shown in Fig. 1A; it was known (1) that the *aac-hph* cluster in pWP7b is flanked by these two restriction sites. The sizes of the plasmids were

TABLE 3. Aminoglycoside assays for strains used for hybridization

Strain	Aminoglycoside-modifying activity (cpm)[a]						
	Acetylation				Phosphorylation		
	No drug	Amikacin	Gentamicin C1a	Apramycin	No drug	Amikacin	Hygromycin B
BN8701	757	1,115 (1)	21,184 (75)	28,037 (100)	1,520	1,559 (<1)	6,435 (100)
BN8906	1,298	1,837 (2)	21,454 (74)	28,420 (100)	1,347	1,584 (1)	21,808 (100)
BN4101	1,128	1,720 (2)	20,914 (70)	29,268 (100)	1,589	1,402 (<1)	29,462 (100)
E/7852	1,458	2,043 (2)	23,046 (83)	27,315 (100)	1,759	1,874 (<1)	33,993 (100)
E/7874	1,079	1,830 (3)	20,216 (72)	27,552 (100)	1,746	1,796 (<1)	32,105 (100)
E/6776	1,103	2,061 (4)	20,970 (74)	27,982 (100)	1,912	1,695 (<1)	37,556 (100)
E/6734	1,162	1,961 (3)	20,728 (75)	27,397 (100)	2,069	2,085 (<1)	36,560 (100)
E/7343	1,973	4,339 (9)	21,090 (73)	28,250 (100)	1,849	1,907 (1)	5,756 (100)
86061324	1,758	1,899 (<1)	45,196 (71)	62,640 (100)	3,685	4,578 (2)	49,084 (100)

[a] Amikacin was used as a negative control; a preparation containing no drugs was used as a blank. The percent activity relative to apramycin (for acetylation) or hygromycin B (for phosphorylation) as substrates is given in parentheses. The "no drug" value was subtracted in calculating percent activities.

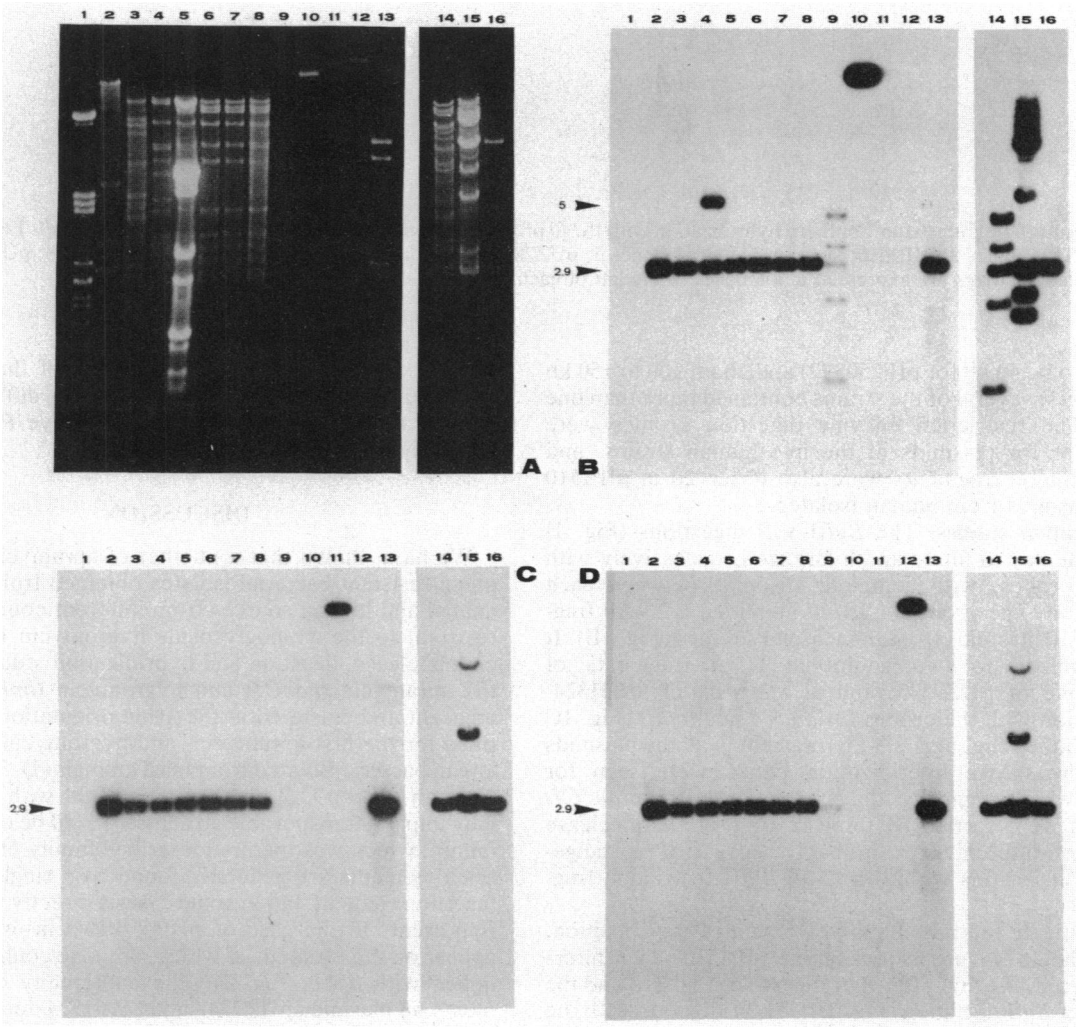

FIG. 1. Agarose gel electrophoresis after double digestion with *Sal*I-*Bgl*II (A) and corresponding autoradiograms after Southern hybridization with the probes for IS*140* (B), *aacC4* (C), and *hphB*(D). Lanes 1, Bacteriophage λ digested by *Pst*I; lanes 2, pIP1508; lanes 3, pIP1509; lanes 4, pIP1510; lanes 5, pUZ7852 (several plasmids?); lanes 6, pUZ7874; lanes 7, pUZ6776; lanes 8, pUZ6734; lanes 9, pUZ7343; lanes 10, probe for IS*140*; lanes 11, probe for *aacC4*; lanes 12, probe for *hphB* (these three probes were deposited on the gel just before the end of the run); lanes 13, pWP7b (two extra restriction bands appear in comparison with lanes 16, probably because of an extra *Sal*I site including a methylated thymine for which digestion was partial); lanes 14, pUZ7343 (the amount of DNA used was five times more than that used in lanes 9); lanes 15, 86061324 (several plasmids); lanes 16, pWP7b. Molecular sizes are expressed in kilobases to the left of panels B through D.

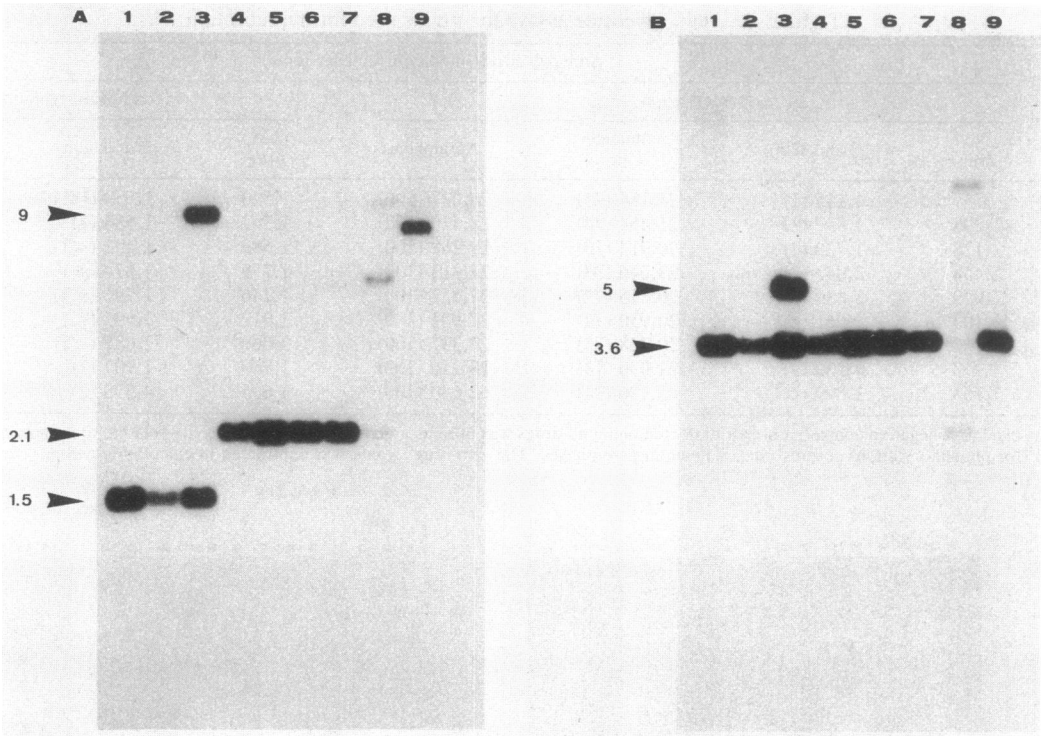

FIG. 2. Autoradiograms after Southern hybridization with IS*140* probe. (A) Digestion with *Pst*I; (B) digestion with *Sal*I. Lanes 1, pIP1508; lanes 2, pIP1509; lanes 3, pIP1510; lanes 4, pUZ7852; lanes 5, pUZ7874; lanes 6, pUZ6776; lanes 7, pUZ6734; lanes 8, pUZ7343; lanes 9, pWP7b. Molecular sizes are expressed in kilobases to the left of each panel.

estimated to be 40 kb for pIP1508 (2) and about 100 to 150 kb for the others; several of the strains contained more than one plasmid. The restriction enzyme digestion profiles were quite similar for plasmids of the five human strains, and several common fragments were also observed in pIP1510 and the plasmids from human isolates.

Hybridization studies. The *Bgl*II-*Sal*I digestions (Fig. 1) were transferred to filters and hybridized successively with the three probes, with complete stripping between each hybridization. The probe for IS*140* identified a 2.9-kb fragment in all 10 plasmids from the various sources (Fig. 1B). It also hybridized to two additional DNA fragments of pIP1510, five of pUZ7343, and at least six of 86061324. Hybridization with the probes for *aacC4* and *hphB* (Fig. 1C and D) identified the same 2.9-kb fragment in all the plasmids studied. Two extra hybridization bands were seen for 86061324, which suggested that in this strain three *aacC4-hphB* pairs were carried by three restriction endonuclease fragments of different sizes, probably because of rearrangements and insertions within the *Sal*I-*Bgl*II restriction fragments.

To investigate the possible involvement of transposition, we used the IS*140* probe to analyze pIP1510. Two filters were prepared, the first after *Pst*I digestion (Fig. 2A) and the second after *Sal*I digestion (Fig. 2B). Hybridization with the IS*140* probe indicated the same number of positively hybridizing fragments as hybridization with *Bgl*II-*Sal*I digestion. In order to explain this feature, we propose three possible configurations for pIP1510 with respect to positions of the two IS*140* fragments, which are probably completely independent but could also be directly or inversely repeated. Schemes illustrating these hybridization studies are shown in Fig. 3. The data in Fig. 2 also suggest that, in the cases examined, the resistance gene clusters of the animal- and human-derived plasmids were inserted in different regions, as indicated by the sizes of the respective *Pst*I fragments which hybridize with the IS*140* probe.

DISCUSSION

We have shown that a number of apramycin- and gentamicin-resistant bacterial isolates obtained from a variety of animal and human sources from different countries are also resistant to the aminoglycoside hygromycin B. Restriction endonuclease digestion and hybridization studies show that the apramycin (*aacC4*) and hygromycin (*hphB*) genes are adjacent and clustered in the same orientation as that identified for the first apramycin- and hygromycin-resistant isolate in bacteria isolated from farm animals (1). The two genes form an operon (10) and are associated with IS*140*, which may imply a transposable structure. IS*140* belongs to the IS*6* family, which was the first described family of this type (6). Such elements are generally found as a single copy of the insertion element, but in some cases a directly repeated copy is present. In the case of pIP1510 two insertion elements appear on the plasmid, of which only one could carry *aacC4* linked with *hphB* (Fig. 3). The multiplicity of IS*140* fragments on plasmids pUZ7343 and 86061324 does not permit a simple analysis of the question of transposition of the resistance gene operon.

It is probable that the strong selection pressure from the use of apramycin and hygromycin B in animals led to the emergence of this type of resistance in animals. Subsequently, the appearance in human isolates was probably due to selection by gentamicin used in both human and veterinary medicine. The transfer of antibiotic resistance genes (or

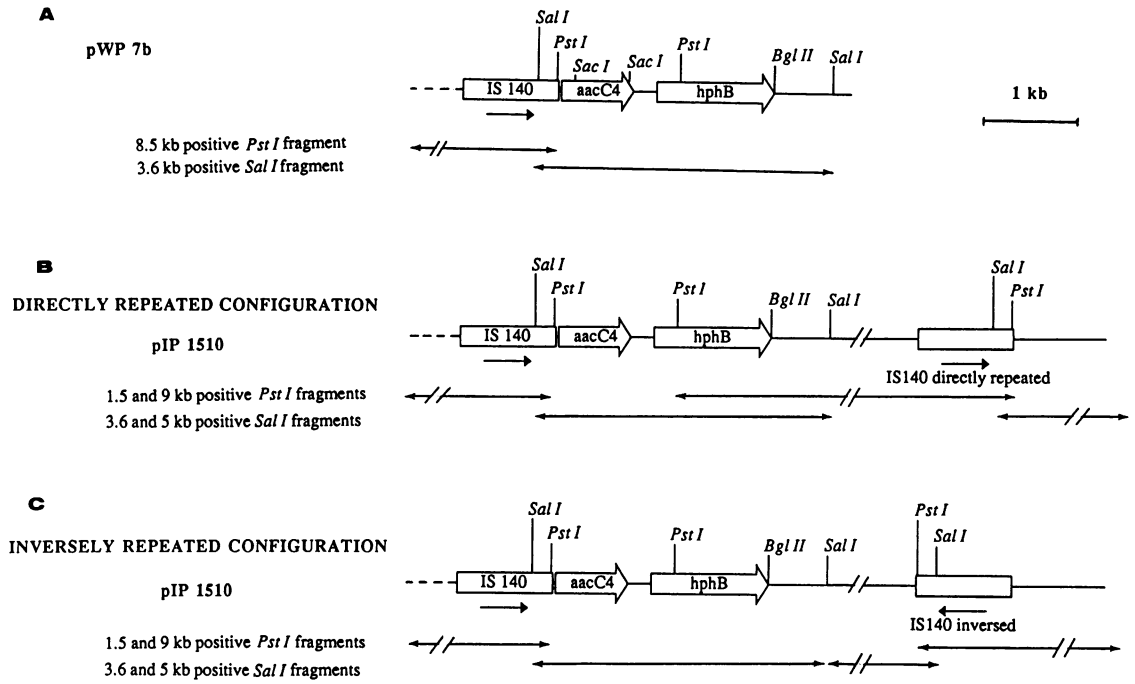

FIG. 3. The part of pWP7b which includes the *SalI-BglII* fragment (A), indicating two of the possible configurations of pIP1510 with either directly repeated (B) or inversely repeated (C) IS*140* fragments. The DNA fragments identified by hybridization with the IS*140* probe are represented under each scheme. This diagram indicates two possible transposable configurations, but the insertion element may also be completely independent.

plasmids) in bacteria from animals to humans has now been demonstrated in several cases (3, 12). We provide plausible evidence for another example, which is of interest since two new antibiotic resistance genes in the form of an operon can be shown to be present in human clinical isolates, although previously they were found only in animals. The mechanism of this transfer is presently unknown; the apramycin- and hygromycin B-resistant human and animal isolates analyzed in this study came from quite different geographical locations. While the *hphB* gene is of no significance in humans, since hygromycin B and its derivatives are used strictly for veterinary purposes, the *aacC4* gene, which encodes resistance to a variety of aminoglycosides, including gentamicin and tobramycin, is an unwanted addition to the known armory of resistance determinants in human isolates. Clearly, the organization of resistance genes on plasmids and transposons can determine which resistance characters are transferred. In the case we describe for apramycin (or gentamicin) and hygromycin B, the association of the two genes in an operon (10) controlled by a single regulatory element guarantees their joint transfer and function. The evolution of multiple-resistance gene operons merits further investigation.

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LITERATURE CITED

1. **Brau, B., U. Pilz, and W. Piepersberg.** 1984. Genes for gentamicin-(3)-N-acetyltransferase III and IV: nucleotide sequence of the AAC(3)-IV gene and possible involvement of an IS 140 element in its expression. Mol. Gen. Genet. **193**:179–187.
2. **Chaslus-Dancla, E., J.-L. Martel, C. Carlier, J.-P. Lafont, and P. Courvalin.** 1986. Emergence of aminoglycoside 3-*N*-acetyltransferase IV in *Escherichia coli* and *Salmonella typhimurium* isolated from animals in France. Antimicrob. Agents Chemother. **29**:239–243.
3. **Corpet, D.** 1989. Les microbes font de la résistance. Recherche **20**:1388–1390.
4. **Davies, J., and S. O'Connor.** 1978. Enzymatic modification of aminoglycoside antibiotics: 3-*N*-acetyltransferase with broad specificity that determines resistance to the novel aminoglycoside apramycin. Antimicrob. Agents Chemother. **14**:69–72.
5. **Ericsson, H. M., and J. C. Scherris.** 1971. Antibiotic sensitivity testing. Report of an international collaborative study. Acta Pathol. Microbiol. Scand. Sect. B Suppl. **217**:1–90.
6. **Galas, D. J., and M. Chandler.** 1989. Bacterial insertion sequences, p. 109–162. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
7. **Gritz, L., and J. Davies.** 1983. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene **23**:179–188.
8. **Haas, M. J., and J. E. Dowding.** 1975. Aminoglycoside modifying enzymes. Methods Enzymol. **43**:611–628.
9. **Hedges, R. W., and K. P. Shannon.** 1984. Resistance to apramycin in *Escherichia coli* isolated from animals: detection of a novel aminoglycoside modifying enzyme. J. Gen. Microbiol. **130**:473–482.
10. **Kastner, K. R., S. G. Burgett, N. R. Rao, and T. D. Ingolia.** 1983. Analysis of a bacterial hygromycin B resistance gene by transcriptional and translational fusions and by DNA sequencing. Nucleic Acids Res. **11**:6895–6911.
11. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

12. **O'Brien, T. F., J. D. Hopkins, E. S. Gilleece, A. A. Medeiros, R. L. Kent, and B. O. Blackburn.** 1982. Molecular epidemiology of antibiotic resistance in *Salmonella* from animals and human beings in the United States. N. Engl. J. Med. **307**:1–6.
13. **Piepersberg, W., B. Brau, and J. Davies.** 1983. High frequency transduction of R-factor encoded gentamicin resistance by bacteriophage P1Cm. J. Antibiot. **36**:588–594.
14. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
15. **Watanabe, T., and T. Fukasawa.** 1961. Episome-mediated transfer of drug resistance in *Enterobacteriaceae*. I. Transfer of resistance factors by conjugation. J. Bacteriol. **81**:669–678.
16. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.