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PCR Mapping of Integrons Reveals Several Novel Combinations of Resistance Genes

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The integron is a new type of mobile element which has evolved by a site-specific recombinational mechanism. Integrons consist of two conserved segments of DNA separated by a variable region containing one or more genes integrated as cassettes. Oligonucleotide probes specific for the conserved segments have revealed that integrons are widespread in recently isolated clinical bacteria. Also, by using oligonucleotide probes for several antibiotic resistance genes, we have found novel combinations of resistance genes in these strains. By using PCR, we have determined the content and order of the resistance genes inserted between the conserved segments in the integrons of these clinical isolates. PCR mapping of integrons can be a useful epidemiological tool to study the evolution of multiresistance plasmids and transposons and dissemination of antibiotic resistance genes.

The dissemination of antibiotic resistance genes among bacterial strains is an increasing problem in infectious diseases. Many antibiotic resistance genes are located on plasmids and on transposons, enabling their transfer among a variety of bacterial species. In recent years, a third mechanism of resistance gene dissemination has been discovered. It involves a DNA element that mediates the integration of resistance genes by a site-specific recombinational mechanism. This novel DNA element, now called an integron (37), is found either as part of transposons of the Tn*21* family or independently on several groups of broad-host-range plasmids. Integrons possess two conserved segments separated by a variable region which includes integrated antibiotic resistance genes or cassettes of unknown function. The 5′ conserved segment contains the *int* gene, which encodes a polypeptide of 337 amino acids that has been shown to be homologous to other members of the integrase family (26), and, on the opposite strand, a common promoter region, *P1-P2*, directed toward the site of integration (21). Since most genes inserted into integrons lack their own promoters, they are expressed from the common promoter region as a resistance operon. The 3′ conserved segment contains the *qacEΔ1* (28) and *sulI* (37, 39) genes and an open reading frame (ORF), orf5 (37). The *qacEΔ1* and *sulI* genes determine resistance to ethidium bromide and quaternary ammonium compounds (28) and to sulfonamide, respectively.

The general structure of integrons is shown in Fig. 1. At the downstream end of each resistance gene cassette inserted in the variable region of integrons, there is a short imperfect inverted repeat element called the 59-base element (6, 13, 37). Each of the inserted genes has its own version of this element. In plasmid pVS1, which possesses the 5′ and 3′ conserved segments but no inserted gene between the conserved segments, there is no 59-base element (1). These 59-base elements are known to be important in the recombination events observed in the evolution of integrons (12). A model for gene insertion in which circular gene cassettes are inserted individually via a single site-specific recombination event has been proposed and verified experimentally (7, 8). Site-specific insertion of gene cassettes thus represents a further mechanism which contributes to the evolution of the plasmids and transposons of gram-negative bacteria.

The plasmids and transposons whose study permitted the discovery of integrons were isolated 15 to 20 years ago. In the current study, recent clinical strains of members of the family *Enterobacteriaceae* and pseudomonads were used to study the role of integrons as vehicles for antibiotic resistance genes. First, by using oligonucleotide probes, we determined the frequency of the presence of the 5′ and 3′ conserved segments of integrons and showed novel combinations of resistance genes in these bacteria. By using PCR, we determined the content and order of the antibiotic resistance genes inserted between the conserved segments in their integrons. PCR analysis of integrons enables us to study the evolution of antibiotic resistance gene dissemination.

(These results were presented in part at the 92nd Annual Meeting of the American Society for Microbiology, New Orleans, La., 1992 [20].)

MATERIALS AND METHODS

Bacterial strains and plasmids. The clinical bacteria used (*Enterobacteriaceae* and pseudomonads) were collected because of their aminoglycoside resistance by G. Miller's group at Schering-Plough Corporation, New Jersey, over the period from 1972 to 1990. The strains were *Salmonella typhimurium* 90123101; *Enterobacter cloacae* 880516588 and 88040794; *Klebsiella pneumoniae* 880516154, OADLER17, 88111811, and 76091601; *Proteus mirabilis* 88071820; *Providencia rettgeri* 75082824 and 76012805; *Pseudomonas fluorescens* 84070206 and 87090481; *Pseudomonas aeruginosa* OSTONE130E, 75081109, OTRAVERS1B, 73101501, 72072401, 75022119, 76120702, and 84061101; *Enterobacter aerogenes* 87122177 and 87122176; *Escherichia coli* 72091801, 73110901, OLA290R55B, 87061002, 87061001, and 87041704; *Salmonella enteritidis* 76061701; *Serratia liquifaciens* 87042862; and *Serratia marcescens* 82041944, 88051616, 75041111, 88050909, 82041946, and 82041947. In the text, we have used the last three numbers to represent the Schering numbers. Plasmids used as positive amplification controls were RIP71a (In2 in transposon Tn*21*) (34), RGN238 (In8 in transposon Tn*2603*) (51), pCER100 (In4 in transposon Tn*1696*) (31), and NR79 (In21 in transposon Tn*2424*) (23).

Growth conditions. Resistant clinical bacteria were cultured in YT (yeast extract plus tryptone; Difco, Detroit, Mich.) medium (32) supplemented with

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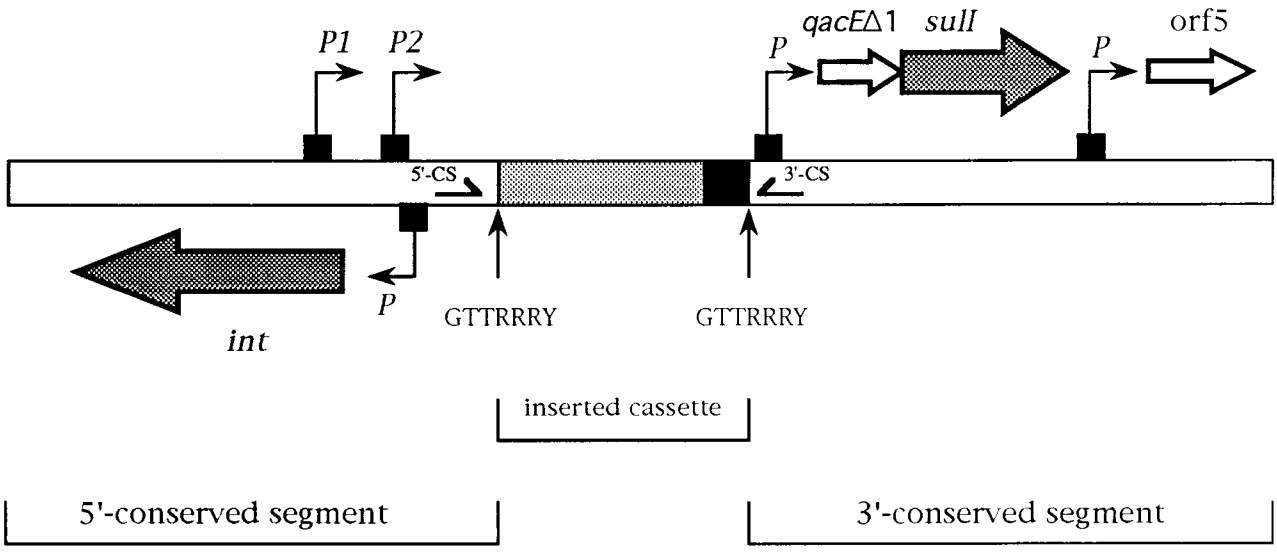

FIG. 1. General structure of integrons. The arrows show the direction of transcription. The location and orientation of different promoters are shown. The sequence GTTRRRY is the integron's crossover point for integration of gene cassettes. The 5′-CS and 3′-CS oligonucleotides are specific to the 5′ and 3′ conserved segments, respectively. They were used as probes for colony hybridization and as primers for PCR analysis of integrons. One inserted cassette is shown, with its associated 59-base element (37) indicated by the black bar.

one or more of the following antibiotics: amikacin (25 μg/ml), ampicillin (25 μg/ml), gentamicin (25 μg/ml), kanamycin (25 μg/ml), streptomycin (25 μg/ml), sulfonamide (25 μg/ml), tobramycin (25 μg/ml), or trimethoprim (25 μg/ml), as appropriate.

Colony hybridization. The colony hybridization technique has been described elsewhere (25) and was used with the following modifications: prehybridization and hybridization were done in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.25% powdered skim milk (Carnation). The hybridization temperature was estimated with the following equation (18): $T_h = 4(G+C) + 2(A+T) - 5$. The resistance gene probes used were specific for the following antibiotic resistance genes: sulfonamide resistance gene *sulI*; β-lactam resistance genes *oxa2* and *pse2*; aminoglycoside resistance genes *aac(6′)-Ia* (*aacA1*), *aac(6′)-Ib* (*aacA4*), *aac(3)-Ia* (*aacC1*), *aac(3)-IIa* (*aacC2*), *aac(3)-IVa* (*aacC4*), *ant(3″)-Ia* (*aadA1*), *ant(3″)-Ib* (*aadA2*), and *ant(2″)-Ia* (*aadB*); trimethoprim resistance genes *dhfrI*, *dhfrIIc*, and *dhfrV*; and tetracycline resistance genes *tetB* and *tetC* (Table 1). The posthybridization washes described by Ouellette and Roy (25) were replaced by three 10-min washes at T_h. Autoradiography was done with Kodak X-Omat AR film.

PCR amplification. Resistant bacteria were grown in 4 ml of brain heart infusion (BHI; Difco)–10% glycerol in the presence of a selective antibiotic at 37°C overnight. Then, 200 μl of the culture was added to 800 μl of distilled water and boiled for 10 min. The bacterial suspension was then centrifuged at 12,000 × *g* for 2 min, and the supernatant was used for PCR.

PCRs were carried out in 100-μl volumes containing 10 μl of 10× PCR buffer

TABLE 1. Oligonucleotides for colony hybridization and for PCR analysis of integrons

Primer or probe	Nucleotide sequence (5′ to 3′)	Position in published sequence (reference)	Accession no.[a]
5′-CS	GGC ATC CAA GCA GCA AG	1190–1206 (1)	M73819
3′-CS	AAG CAG ACT TGA CCT GA	1342–1326 (1)	M73819
sulI	TGA AGG TTC GAC AGC AC	1463–1447 (39)	X12869
aac(6′)-Ia	TAA TTG CTG CAT TCC GC	797–781 (44)	M18967
aac(6′)-Ib	TGT GAC GGA ATC GTT GC	432–416 (47)	M23634
aac(3)-Ia	AGC CCG CAT GGA TTT GA	1375–1359 (50)	X15852
aac(3)-Ia-3′	GGC ATA CGG GAA GAA GT	1730–1746 (50)	X15852
aac(3)-IIa	CCT CCG TTA TTG CCT TC	118–102 (48)	X51534
aac(3)-IVa	TCG GCT TTT CGC CAT TC	269–253 (5)	X01385
ant(3″)-Ia	TCG ATG ACG CCA ACT AC	464–448 (16)	X02340
ant(3″)-Ib	TCA ATG ACG CTT AGC AC	457–441 (43)	M11444
		1276–1260 (14)	Z21672
ant(3″)-I-3′	CGC AGA TCA (C/G)TT GGA AG	1128–1144 (16)	X02340
		1121–1137 (43)	M11444
ant(2″)-Ia	CCG CAG CTA GAA TTT TG	1341–1325 (6)	X04555
oxa2	AAC CCG GCA GTC AA	2255–2268 (13)	X06046
pse2	GTA CTC GAA AGA CAC GC	177–161 (17)	J03427
dhfrI	AGC TGT TCA CCT TTG GC	1059–1043 (36)	K00052
dhfrIIc	ACT AGA GTA CTG ACT CC	369–353 (11)	X04128
dhfrV	ATC ACT CCG TTT TTC GC	1349–1333 (39)	X12868
tetB	CAA AAC TTG CCC CTA AC	475–459 (15)	V00611
tetC	GTT GAA GGC TCT CAA GG	679–663 (42)	V01119
tnpR	AGC TCG ACC GTC TTG GC	246–262 (45)	M55547
tem	GGC GTC AAC ACG GGA TA	3904–3920 (42)	V01119

[a] Accession numbers are for the EMBL/GenBank database.

(100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, 1 mg of gelatin per ml), 10 μl of 10× deoxynucleoside triphosphate mix (2 mM each dATP, dCTP, dGTP, and dTTP), 10 μl of each primer stock solution (2.5 pmol of each primer per μl), 30 μl of template DNA, and 30 μl of sterile distilled water. Each reaction mix was covered with 75 μl of mineral oil (22). *Taq* DNA polymerase (Perkin Elmer Cetus, Emeryville, Calif.) was added (1 μl of the 3-U/μl diluted solution) after 12 min at 94°C (hot start method). To amplify the DNA in the thermal cycler, we used a three-step profile: 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 5 min of extension at 72°C for a total of 35 cycles. Five seconds were added to the extension time at each cycle.

Gel electrophoresis and DNA hybridization. To visualize the amplification product, the reaction products were electrophoresed at 100 V for 2 h on a 0.7% agarose gel containing 0.5 μg of ethidium bromide per ml using TBE (32) running buffer. DNA was then transferred to nylon filters by Southern blotting as described by Sambrook et al. (32), except that the transfer-denaturation solution is 1 M NaOH. The nylon filters were prehybridized for 2 h at 37°C in the prehybridization solution, which consisted of 5× SSC, 1% sodium dodecyl sulfate (SDS), 1× Denhardt's solution (50× Denhardt's solution is 10 g of Ficoll, 10 g of polyvinylpyrrolidone, and 10 g of bovine serum albumin per liter), 0.1% sodium PP_i, and 100 μg of sonicated salmon sperm DNA per ml. The filters were then incubated overnight at T_h in hybridization solution (same as prehybridization solution but without Denhardt's solution) with 0.6×10^7 to 1×10^7 cpm of the labelled probe. The filters were washed at T_h three times in 2× SSC–0.1% SDS for 15 min and twice in 1× SSC–0.1% SDS for 15 min. Autoradiography was done with Kodak X-Omat AR film.

DNA sequencing of PCR products. The PCR-generated fragments were first purified with Nacs-52 Prepac ion-exchange resin minicolumns purchased from Gibco BRL Canada. The double-stranded PCR material was then sequenced by the dideoxy chain termination method (33) except that the annealing of primer with template was done by a snap-cooling procedure (19).

Computer analysis. Sequences chosen for oligonucleotides were first tested by computer analysis against the GenBank and EMBL databases with the FIND program (9) to avoid unwanted hybridization to known sequences. The nucleotide sequence of the PCR amplification fragments from the *Proteus mirabilis* (820) strain was analyzed with the Genetics Computer Group software (9).

RESULTS AND DISCUSSION

Frequency of the presence of the integron sequence context in resistant clinical strains. The results of the hybridization experiments are shown in Table 2. Hybridization with the 5′-CS and 3′-CS probes, specific to the 5′ and 3′ conserved segments of integrons, respectively (Fig. 1), showed that both probes hybridized to 26 of 35 clinical strains (nearly 75%). These results indicate that integron-related sequences are widely disseminated among aminoglycoside-resistant strains of *Enterobacteriaceae* and pseudomonads. When the hybridization results obtained with the probes specific for the conserved segments were compared with those obtained with the sulI probe, we observed that 24 of 26 strains that were positive for the 5′-CS and 3′-CS probes (>90%) hybridized with the sulI probe. In a previous study (30) that investigated plasmid-mediated sulfonamide resistance, it was shown that 100% of the clinical isolates harboring *sulI* also hybridized to a probe for the integrase gene. These results indicated that *sulI* is strongly linked to integrons. Also, *sulI* was present in 18 of 19 previously mapped integrons (1).

Occurrence of antibiotic resistance genes in aminoglycoside-resistant clinical bacteria. The most common streptomycin-spectinomycin resistance genes are *ant(3″)-Ia* and *ant(3″)-Ib*, two genes that are 88% identical (2, 29). We found that 20 of 28 streptomycin-resistant isolates gave a positive signal with one or the other of the probes for these genes. The presence of *ant(3″)-Ia* in integron In2 of transposon Tn*21*, as well as in Tn*7*, may favor its transfer among a variety of bacterial species.

The probe results in Table 2 show that the aminoglycoside resistance genes *ant(2″)-Ia*, *aac(3)-Ia*, *aac(3)-IIa*, and *aac(6′)-Ib* were also widespread among the Schering collection of clinical isolates. These genes determine resistance to several antibiotics, such as gentamicin, tobramycin, kanamycin, amikacin, and netilmicin. Only a few clinical strains hybridized with the aac(6′)-Ia probe. These results agree with observations by Shaw et al. (35).

Several isolates contain two or three of the aminoglycoside resistance genes in the same bacterium. Some of these combinations, for example *ant(2″)-Ia*, *aac(3)-Ia*, and *aac(6′)-Ib*, have not previously been found in integrons, although they occur individually in previously described integrons. This suggested the presence of multiresistance integrons in these strains. Of the preceding genes, only *aac(3)-IIa* is not known to occur in integrons, but rather is flanked by IS*140*s, forming a possible transposon.

The integron-associated β-lactamase genes *oxa2* and *pse2* were not widely disseminated among the strains tested. These genes are rare in comparison to *tem1*, present in more than 75% of ampicillin-resistant clinical *E. coli* strains (49).

The trimethoprim resistance gene *dhfrI* was present in a few clinical strains. While *dhfrI* is found primarily in the transposon Tn*7*, it is also found in integrons such as those of pLMO150 and pLMO229 (41). None of the strains tested carried the integron-associated *dhfrIIc* and *dhfrV* genes.

The *tetB* gene was found in several clinical isolates, while none of the strains carried *tetC*. The *tetB* gene is borne on Tn*10* and is not found in integrons.

PCR mapping of integrons. By using PCR, we determined the content and order of the antibiotic resistance genes inserted between the conserved segments in the integrons of several clinical isolates. First, by using the 5′-CS and 3′-CS primers, we amplified the variable regions of the integrons. Then, the antibiotic resistance genes inserted between the conserved segments were determined by making a Southern blot of the PCR product and hybridizing to probes that are specific to resistance genes known to occur in each strain (Table 2). Since the *ant(3″)-I* genes *ant(3″)-Ia* and *ant(3″)-Ib* are often found in integrons, primers near the ends of the *ant(3″)-I* genes [primers ant(3″)-Ia and ant(3″)-Ib for the upstream end of the corresponding genes and ant(3″)-I-3′ for the downstream end of either gene] were used in combination with primers for the conserved segments. These PCRs yielded products which included the sum of genes inserted upstream and downstream of *ant(3″)-I*. The last step of mapping was to determine the gene order in integrons. This was done by using primers located at the extremities of the inserted resistance genes in combination with those specific to the conserved segments. The general scheme for detection of integrons is shown in Fig. 2.

As positive amplification controls, we used plasmids containing integrons whose gene content and order have been confirmed by DNA sequencing. First, we used DNA from RIP71a, which contains only *ant(3″)-Ia* inserted between the conserved segments. PCR amplification from this clone yielded a product of 1 kb. Also, we used DNA from RGN238, in which *oxa1* and *ant(3″)-Ia* are inserted and which yielded a product of 2 kb; from pCER100, in which *aac(3)-Ia*, orfE, *ant(3″)-Ib*, and *cmlA* are inserted and which yielded a product of 3.5 kb; and from NR79, in which seven cassettes are inserted and which yielded a PCR product of 5.5 kb. Figure 3 shows the PCR amplification of the variable regions for these positive controls.

Figure 4 shows the PCR amplification of the variable regions from some recent clinical isolates. The *K. pneumoniae* 154, *P. aeruginosa* 702, and *E. aerogenes* 177 strains yielded PCR products of 1 kb, while the *E. cloacae* 588 and *S. marcescens* 616, 946, 947, and 909 strains gave products of 1.6 kb. The *S. typhimurium* 101, *E. coli* 801, and *P. mirabilis* 820 strains yielded PCR products of 2, 3, and 3.5 kb, respectively. These results showed that all the strains tested contain an integron which possesses one or more inserted genes, suggesting the presence of multiresistance integrons in these clinical strains.

The integrons mapped from the *K. pneumoniae* 154, *P. aeruginosa* 702, and *E. aerogenes* 177 strains were identical. The

TABLE 2. Hybridization to strains supplied by Schering Corporation

Bacterial strains (abbreviated Schering strain no.)	Phenotypes[a]	5′ CS	3′ CS	sulI	aac6′Ia	aac6′Ib	aac3Ia	aac3IIa	aac3IVa	ant3″Ia	ant3″Ib	ant2″Ia	oxa2	pse2	tetB	dhfrI
E. cloacae (588)	AAC(6′)-I, AAC(3)-II, ANT(3″), ANT(2″), APH(3′)-I	+	+	+	−	+	−	+	−	+	−	+	−	−	−	+
E. cloacae (794)	AAC(6′)-I, ANT(3″), ANT(2″), APH(3′)-I	+	+	+	−	+	−	−	−	+	−	+	−	−	−	+
K. pneumoniae (601)	AAC(3)-II	+	+	+	−	−	−	+	−	+	−	−	−	−	−	−
K. pneumoniae (R17)	APH(3′)-II	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−
K. pneumoniae (811)	AAC(6′)-I, ANT(3″)	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
K. pneumoniae (154)	AAC(6′)-I, AAC(3)-II, ANT(3″), ANT(2″), APH(3′)-I	+	+	+	−	−	−	+	−	+	−	−	−	−	−	−
P. mirabilis (820)	AAC(3)-I, AAC(3)-II, ANT(3″), APH(3′)-I	+	+	+	−	−	+	+	−	−	+	−	−	−	−	+
P. rettgeri (824)	AAC(2′)	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
P. rettgeri (805)	AAC(2′)	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−
P. fluorescens (206)	AAC(3)-IV, AAC(2′), APH(3′)-IV	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
P. fluorescens (481)	AAC(6′)-I, ANT(2″)	+	+	+	−	+	−	−	−	−	+	+	−	−	−	−
P. aeruginosa (30E)	AAC(3)-I	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−
P. aeruginosa (109)	AAC(3)-I	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−
P. aeruginosa (S1B)	AAC(3)-III	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
P. aeruginosa (501)	AAC(6′)-II	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−
P. aeruginosa (401)	AAC(6′)-II	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
P. aeruginosa (119)	ANT(2″)	+	+	+	−	−	−	−	−	−	−	+	−	+	−	−
P. aeruginosa (702)	ANT(2″)	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−
P. aeruginosa (101)	ANT(4′)-II	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−
E. aerogenes (177)	AAC(6′)-I, AAC(3)-II, ANT(3″), ANT(2″)	+	+	−	−	+	−	+	−	+	−	−	−	−	−	−
E. aerogenes (176)	AAC(3)-I, AAC(6′)-I, ANT(3″), ANT(2″)	+	+	+	−	+	+	−	−	+	−	−	−	−	−	−
E. coli (801)	AAC(3)-I	+	+	+	−	−	+	−	−	+	−	−	−	−	−	−
E. coli (901)	AAC(6′)-I	+	+	+	+	−	−	−	−	−	−	−	−	−	+	−
E. coli (55B)	ANT(2″)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
E. coli (002)	AAC(6′)-I, ANT(2″)	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
E. coli (001)	ANT(2″)	+	+	+	−	−	−	−	−	+	−	−	−	−	+	−
E. coli (704)	ANT(3″)	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
S. enteritidis (701)	AAC(3)-IV	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
S. liquifaciens (862)	AAC(3)-II	−	+	+	−	−	−	+	−	−	−	−	−	−	−	−
S. marcescens (944)	AAC(3)-II	+	+	−	−	−	+	−	−	+	−	−	−	−	−	−
S. marcescens (616)	AAC(3)-II, ANT(3″), ANT(2″), APH(3′)-I	+	+	+	−	−	−	+	−	+	−	+	−	−	−	−
S. marcescens (111)	AAC(6′)-I	+	+	+	−	−	+	−	−	+	−	−	−	−	−	−
S. marcescens (909)	AAC(6′)-I, AAC(3)-I, ANT(3″), ANT(2″), APH(3′)-I	+	+	+	−	+	+	−	−	+	−	+	−	−	−	+
S. marcescens (946)	ANT(2″)	+	+	+	−	−	−	−	−	−	−	+	+	−	−	−
S. marcescens (947)	ANT(2″)	+	+	+	−	−	+	−	−	−	−	+	+	−	−	−

[a] Data on the resistance profiles (phenotype) of the bacteria for sulfonamide, β-lactam, tetracycline, and trimethoprim were not available. AAC(6′)-I and AAC(6′)-II, 6′-*N*-acetyltransferase; AAC(3)-I, AAC(3)-II, AAC(3)-III, and AAC(3)-IV, 3-*N*-acetyltransferase; AAC(2′), 2′-*N*-acetyltransferase; ANT(3″), 3″-*O*-adenylyltransferase; ANT(2″), 2″-*O*-adenylyltransferase; ANT(4′)-I, 4′-*O*-adenylyltransferase; APH(3′)-I, APH(3′)-II, APH(3′)-IV, and APH(3′)-VI, 3′-*O*-phosphotransferase.

ant(3″)-Ia gene was found between the conserved segments of the integrons of these strains by PCR with the 5′-CS and 3′-CS primers in combination with primers for the beginning and the end of *ant(3″)-Ia* [ant(3″)-Ia and ant(3″)-I-3′ primers]. As shown in Table 3, the sizes of the bands obtained [5′-CS and ant(3″)-Ia primers, 170 bp; ant(3″)-I-3′ and 3′-CS primers, 180 bp] corresponded to those predicted by joining the published sequences of the cassettes and conserved sequences at the specific recombination sites (168 and 176 bp, respectively). Also, the presence of the streptomycin-spectinomycin resistance gene was confirmed by hybridization to the specific ant(3″)-Ia probe.

For the *E. cloacae* 588 and *S. marcescens* 616 strains, we found *ant(2″)-Ia* upstream of *ant(3″)-Ia*. Integrons of these strains were mapped by using different combinations of primer pairs (Table 3). In all cases, the sizes of the bands observed corresponded with those predicted. The *ant(2″)-Ia* gene is the most common gene encoding 2″-*O*-adenylyltransferase activity [ANT(2″)-I] (35). This gene is widespread among all gram-negative bacteria, especially *Serratia* species.

PCR mapping of the *S. marcescens* 946 and 947 strains showed a combination of antibiotic resistance genes, *ant(2″)-Ia* and *oxa2*, in that order (Table 3), which have not previously been found together in the same integron. All the β-lactamase genes mapped in integrons have been found in the first position in the antibiotic resistance operon with the exception of In1 in plasmid R46, in which two identical copies of *oxa2* are present (38).

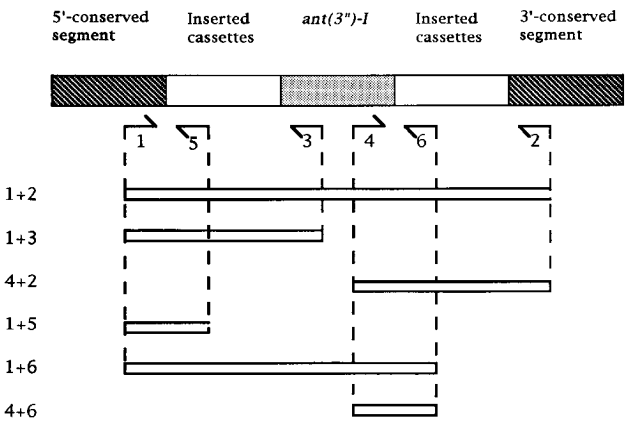

FIG. 2. General scheme for detection of integrons. PCR mapping was done with primers in the conserved segments (primers 1 and 2) and near the ends of the *ant(3″)-I* genes (primers 3 and 4). To determine the gene order in integrons, we used primers for the beginning of various resistance genes (represented by 5 and 6) in combination with those specific to the conserved segments or the *ant(3″)-I* genes. Primer 1, 5′-CS primer; primer 2, 3′-CS primer; primer 3, ant(3″)-Ia or ant(3″)-Ib primer; primer 4, ant(3″)-I-3′ primer. Primer 4 [ant(3″)-I-3′ primer] is the same for *ant(3″)-Ia* and *ant(3″)-Ib*.

The integron of *S. marcescens* 909 contained *dhfrI* and *ant(3″)-Ia*. Their presence was revealed by using primer pairs 5′-CS and dhfrI, 5′-CS and ant(3″)-Ia, and ant(3″)-I-3′ and 3′-CS (Table 3). The *dhfrI* and *ant(3″)-Ia* genes are also found in integron In18 in plasmid pLMO229. The same two genes also occur in Tn*7*, where they are separated by *sat*, a gene encoding streptothricin acetyltransferase (40).

For the *S. typhimurium* 101 strain, there were also two genes inserted between the conserved segments, *aac(6′)-Ib* and *ant(3″)-Ia* (Table 3). In In23 of pMG7, the former gene is well expressed in *Pseudomonas* strains (10) but is poorly expressed

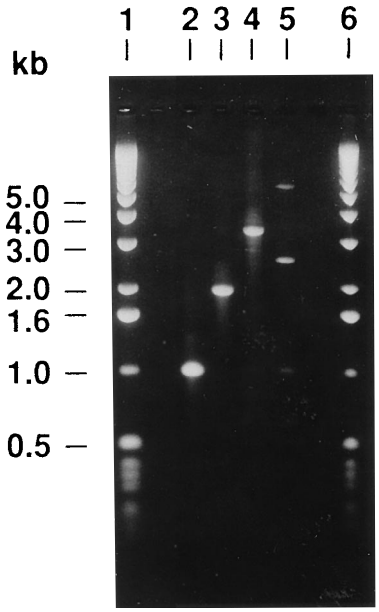

FIG. 3. PCR amplification of positive controls. The PCR products were separated by electrophoresis in 0.7% agarose. Lanes 1 and 6, 1-kb DNA ladder; lane 2, RIP71a (In2); lane 3, RGN238 (In8); lane 4, pCER100 (In4); lane 5, NR79 (In21). For the control NR79 (lane 5), the 1.0-kb and 2.5-kb bands are shorter fragments representing possible rearrangements that have lost some of the genes found in the variable region of integron In21 in Tn*2424*.

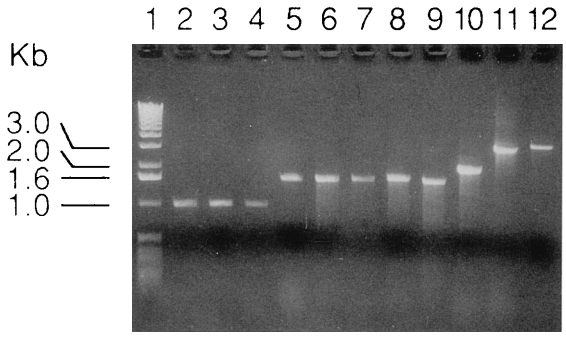

FIG. 4. PCR amplification, using the 5′-CS and 3′-CS primers, of variable regions of integrons from recent clinical isolates. The PCR products were separated by electrophoresis in 0.7% agarose. Lane 1, 1-kb DNA ladder; lane 2, *K. pneumoniae* 154; lane 3, *P. aeruginosa* 702; lane 4, *E. aerogenes* 177; lane 5, *E. cloacae* 588; lane 6, *S. marcescens* 616; lane 7, *S. marcescens* 946; lane 8, *S. marcescens* 947; lane 9, *S. marcescens* 909; lane 10, *S. typhimurium* 101; lane 11, *E. coli* 801; lane 12, *P. mirabilis* 820.

in *E. coli* K strains (4). In Tn*1331*, an element in which three antibiotic resistance genes [*aac(6′)-Ib*, *ant(3″)-Ia*, and *oxa9*] are inserted into Tn*3* (46), AAC(6′)-Ib is a translational fusion with the TEM β-lactamase and is well expressed in *E. coli* (47). It will be interesting to investigate the expression of *aac(6′)-Ib* in relation to its position within the antibiotic resistance operon.

The variable region of the integron found in *E. coli* 801 includes four gene cassettes (Table 3), two of which are the antibiotic resistance genes *aac(3)-Ia* and *ant(3″)-Ib*. Downstream of *aac(3)-Ia*, there is an ORF, orfE, identical to that found in In4 in transposon Tn*1696*. The fourth cassette encodes an ORF whose partial sequence shows no similarity to known genes (3).

For the *P. mirabilis* 820 strain, the gene cassettes inserted between the conserved segments are *aac(3)-Ia*, orfE, *ant(3″)-Ib*, and *cmlA*, the same arrangement as that found in transposon Tn*1696*. As we did not have oligonucleotide probes to detect orfE and *cmlA*, the presence of these cassettes in this integron was revealed by DNA sequencing. First, we amplified the variable region using the 5′-CS and 3′-CS primers, yielding a product of 3.5 kb (Fig. 4). The aac(3)-Ia-3′ primer was used to sequence across the junction of *aac(3)-Ia* and orfE, and the ant(3″)-I-3′ primer was used to obtain sequence across the junction of *ant(3″)-Ib* and *cmlA*. The integron of the *P. mirabilis* 820 strain was mapped as shown in Fig. 5. The sizes of the bands obtained correspond with those predicted from published sequences (Table 3).

Many of the aminoglycoside resistance genes that gave a positive signal in the colony hybridization experiments have been mapped in the integrons of the clinical isolates. However, some genes normally associated with integrons were not found in the integrons mapped in this work. The *aac(6′)-Ib* gene, which can occur in integrons but also in Tn*1331*, did not map within the integrons of the *E. aerogenes* 177, *E. cloacae* 588, and *S. marcescens* 909 strains. Primers specific for *tnpR* (resolvase) and *tem* β-lactamase yielded a product of 3.9 kb from *E. aerogenes* 177 (data not shown), which corresponds to the distance between these primers in Tn*1331*. The content of this PCR product was confirmed by hybridization to the aac(6′)-Ib and ant(3″)-Ia probes, specific for genes found in Tn*1331* (data not shown). For the other strains, PCR amplification with the tnpR and tem primers yielded a product of about 800 bp that corresponds to the transposon Tn*3* alone (data not shown). The precise locations of *aac(6′)-Ib* in the *E. cloacae* 588 and *S. marcescens* 909 strains, as well as *aac(3)-Ia* in the *S. marcescens*

TABLE 3. PCR amplification products

Bacterial strains (abbreviated Schering strain no.)	Primer pair	Length of product (bp)		Hybridization[a]	Gene order (insert region)
		Predicted	Observed		
K. pneumoniae (154), *P. aeruginosa* (702), *E. aerogenes* (177)	5′-CS and 3′-CS	1,009	1,000	ant(3″)-Ia	*ant(3″)-Ia*
	5′-CS and ant(3″)-Ia	168	170		
	ant(3″)-I-3′ and 3′-CS	176	180		
E. cloacae (588), *S. marcescens* (616)	5′-CS and 3′-CS	1,600	1,600	ant(2″)-Ia, ant(3″)-Ia	*ant(2″)-Ia–ant(3″)-Ia*
	5′-CS and ant(2″)-Ia	152	160		
	5′-CS and ant(3″)-Ia	759	750	ant(2″)-Ia	
	ant(3″)-I-3′ and 3′-CS	176	180		
S. marcescens (946), *S. marcescens* (947)	5′-CS and 3′-CS	1,620	1,600	ant(2″)-Ia, oxa2	*ant(2″)-Ia–oxa2*
	5′-CS and ant(2″)-Ia	152	170		
S. marcescens (909)	5′-CS and 3′-CS	1,586	1,600	dhfrI, ant(3″)-Ia	*dhfrI–ant(3″)-Ia*
	5′-CS and dhfrI	206	210		
	5′-CS and ant(3″)-Ia	745	750	dhfrI	
	ant(3″)-I-3′ and 3′-CS	176	180		
S. typhimurium (101)	5′-CS and 3′-CS	1,646	2,000	aac(6′)-Ib, ant(3″)-Ia	*aac(6′)-Ib–ant(3″)-Ia*
	5′-CS and aac(6′)-Ib	148	150		
	5′-CS and ant(3″)-Ia	805	800	aac(6′)-Ib	
	ant(3″)-I-3′ and 3′-CS	176	180		
E. coli (801)	5′-CS and 3′-CS	—[b]	3,000	aac(3)-Ia, ant(3″)-Ia	*aac(3)-Ia*–orfE–ORF?–*ant(3″)-Ia*
	5′-CS and aac(3)-Ia	188	200		
	5′-CS and ant(3″)-Ia	—	2,500	aac(3)-Ia	
	aac(3)-Ia-3′ and ant(3″)-Ia	—	1,800		
	aac(3)-Ia-3′ and 3′-CS	—	2,600	ant(3″)-Ia	
	ant(3″)-I-3′ and 3′-CS	176	180		
P. mirabilis (820)	5′-CS and 3′-CS	3,393	3,500	aac(3)-Ia, ant(3″)-Ib	*aac(3)-Ia*–orfE–*ant(3″)-Ib–cmlA*
	5′-CS and aac(3)-Ia	188	200		
	5′-CS and ant(3″)-Ib	1,025	1,000	aac(3)-Ia	
	aac(3)-Ia-3′ and 3′-CS	2,870	3,000	ant(3″)-Ib	
	aac(3)-Ia-3′ and ant(3″)-Ib	483	500		
	ant(3″)-I-3′ and 3′-CS	1,726	1,700		

[a] Gene probes to which the PCR products hybridized.
[b] Since the integron of *E. coli* 801 contains an inserted cassette (ORF) which remains unidentified, the sizes of PCR products were not predicted.

947 and 909 strains, remain unknown. It would be necessary to clone these genes and sequence their environs in order to determine whether they can occur in other elements.

The *ant(2″)-Ia* gene did not map within the integron of the *S. marcescens* 909 strain, but this gene may be carried by a transposon such as Tn*732* (24). Similarly, *dhfrI*, which did not map within the integrons of the *E. cloacae* 588 and *P. mirabilis* 820 strains, can be supposed to be on Tn*7*, where it occurs most frequently (40).

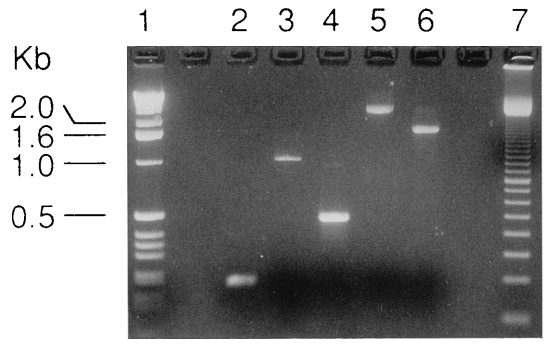

FIG. 5. PCR amplification from *P. mirabilis* 820. The PCR products were separated by electrophoresis through 1.5% agarose. Lane 1, 1-kb DNA ladder; lanes 2 to 6, *P. mirabilis* 820 with: lane 2, 5′-CS and aac(3)-Ia; lane 3, 5′-CS and ant(3″)-Ib; lane 4, aac(3)-Ia-3′ and ant(3″)-Ib; lane 5, aac(3)-Ia-3′ and 3′-CS; and lane 6, ant(3″)-I-3′ and 3′-CS; lane 7, 100-bp DNA ladder.

In summary, integrons are natural expression vectors that permit the insertion of antibiotic resistance genes by a site-specific recombinational mechanism. In this study, by using PCR, we have determined the content and order of the antibiotic resistance genes inserted between the conserved segments in the integrons of recent resistant clinical bacteria. Several of the observed combinations of resistance mechanisms, which were not prevalent 10 years ago, found in many recent clinical isolates can be explained by the integration of antibiotic resistance genes into integrons. PCR mapping of genes inserted as cassettes into integrons will provide valuable information for studies of gene expression as it relates to the position of these genes within the integrons. It is possible that the 59-base element, a potential stem-loop-forming structure found at the downstream end of each inserted gene cassette, may act as an inefficient terminator of transcription, resulting in diminished expression of a gene inserted in the second, third, etc., position in an antibiotic resistance operon.

The accumulation of resistance genes by integrons is one explanation for the emergence of multiply resistant strains of *Enterobacteriaceae* and pseudomonads. Therefore, PCR mapping of integrons can be a useful epidemiological tool to study the evolution of multiresistance plasmids and transposons and dissemination of these antibiotic resistance genes.

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REFERENCES

1. **Bissonnette, L., and P. H. Roy.** 1992. Characterization of In0 of *Pseudomonas aeruginosa* plasmid pVS1, an ancestor of integrons of multiresistance plasmids and transposons of gram-negative bacteria. J. Bacteriol. **174:**1248–1257.
2. **Bito, A., and M. Susani.** 1994. A revised analysis of *aadA2* gene of plasmid pSa. Antimicrob. Agents Chemother. **38:**1172–1175.
3. **Brassard, S.** Unpublished data.
4. **Brassard, S., L. Bissonnette, and P. H. Roy.** 1988. Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 864.
5. **Braü, B., U. Piltz, and W. Piepersberg.** 1984. Genes for gentamicin-(3)-*N*-acetyltransferases III and IV. I. Nucleotide sequence of the AAC(3)-IV gene and possible involvement of an IS140 element in its expression. Mol. Gen. Genet. **193:**179–187.
6. **Cameron, F. F., D. J. Groot Obbink, V. P. Ackerman, and R. M. Hall.** 1986. Nucleotide sequence of the AAD(2″) aminoglycoside adenylyltransferase determinant *aadB*: evolutionary relationship of this region with those surrounding *aadA* in R538-1 and *dhfrII* in R388. Nucleic Acids Res. **14:**8625–8635.
7. **Collis, C. M., and R. M. Hall.** 1992. Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase. J. Bacteriol. **174:**1574–1585.
8. **Collis, C. M., and R. M. Hall.** 1992. Gene cassettes from the insert region of integrons are excised as covalently closed circles. Mol. Microbiol. **19:**2875–2885.
9. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
10. **Falkiner, F. R., C. T. Keane, M. Dalton, M. T. Clancy, and G. A. Jacoby.** 1977. Cross infection in a surgical ward caused by *Pseudomonas aeruginosa* with transferable resistance to gentamicin and tobramycin. J. Clin. Pathol. **30:**731–737.
11. **Flensburg, J., and S. Steen.** 1986. Nucleotide sequence analysis of the trimethoprim resistant dihydrofolate reductase encoded by R plasmid R751. Nucleic Acids Res. **14:**5933.
12. **Hall, R. M., D. E. Brooks, and H. W. Stokes.** 1991. Site-specific insertion of genes into integrons: role of the 59-base element and determination of the recombination crossover point. Mol. Microbiol. **5:**1941–1959.
13. **Hall, R. M., and C. Vockler.** 1987. The region of the IncN plasmid R46 coding for resistance to β-lactam antibiotics, streptomycin/spectinomycin and sulphonamides is closely related to antibiotic resistance segments found in IncW plasmids and in Tn*21*-like transposons. Nucleic Acids Res. **15:**7491–7501.
14. **Heikkilä, E., M. Skurnik, L. Sundström, and P. Huovinen.** 1993. A novel dihydrofolate reductase cassette inserted in an integron borne on a Tn*21*-like element. Antimicrob. Agents Chemother. **37:**1297–1304.
15. **Hillen, W., and K. Schollmeier.** 1983. Nucleotide sequence of the Tn*10* encoded tetracycline resistance gene. Nucleic Acids Res. **11:**525–539.
16. **Hollingshead, S., and D. Vapnek.** 1985. Nucleotide sequence analysis of a gene encoding a streptomycin/spectinomycin adenyltransferase. Plasmid **13:**17–30.
17. **Huovinen, P., S. Huovinen, and G. A. Jacoby.** 1988. Sequence of PSE-2 β-lactamase. Antimicrob. Agents Chemother. **32:**134–136.
18. **Itakura, K., J. J. Rossi, and R. B. Wallace.** 1984. Synthesis and use of synthetic oligonucleotides. Annu. Rev. Biochem. **53:**323–356.
19. **Kusukawa, N., T. Uemori, K. Asada, and I. Kato.** 1990. Rapid and reliable protocol for direct sequencing of material amplified by the polymerase chain reaction. Biotechniques **9:**66–71.
20. **Larose, C., C. Lévesque, and P. H. Roy.** 1992. Program Abstr. 92nd Annu. Meet. Am. Soc. Microbiol., abstr. H8.
21. **Lévesque, C., S. Brassard, J. Lapointe, and P. H. Roy.** 1994. Diversity and relative strength of tandem promoters for the antibiotic-resistance genes of several integrons. Gene **142:**49–54.
22. **Lévesque, C., and P. H. Roy.** 1993. PCR analysis of integrons, p. 590–594. *In* D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), Diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, D.C.
23. **Meyer, J. F., B. A. Nies, and B. Wiedemann.** 1983. Amikacin resistance mediated by multiresistance transposon Tn*2424*. J. Bacteriol. **155:**755–760.
24. **Nugent, M. E., D. H. Bone, and N. Datta.** 1979. A transposon, Tn*732*, encoding gentamicin/tobramycin resistance. Nature (London) **282:**422–423.
25. **Ouellette, M., and P. H. Roy.** 1986. Analysis by using DNA probes of the OXA-1 β-lactamase gene and its transposon. Antimicrob. Agents Chemother. **30:**46–51.
26. **Ouellette, M., and P. H. Roy.** 1987. Homology of ORFs from Tn*2603* and from R46 to site-specific recombinases. Nucleic Acids Res. **15:**10055.
27. **Parent, R., and P. H. Roy.** 1989. Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 153.
28. **Paulsen, I. T., T. G. Littlejohn, P. Rådström, L. Sundström, O. Sköld, G. Swedberg, and R. A. Skurray.** 1993. The 3′ conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. Antimicrob. Agents Chemother. **37:**761–768.
29. **Rådström, P.** 1991. Ph.D. thesis, University of Uppsala, Uppsala, Sweden.
30. **Rådström, P., G. Swedberg, and O. Sköld.** 1991. Genetic analysis of sulfonamide resistance and its dissemination in gram-negative bacteria illustrate new aspects of R plasmid evolution. Antimicrob. Agents Chemother. **35:**1840–1848.
31. **Rubens, C. E., W. F. McNeill, and W. E. Farrar.** 1979. Transposable plasmid deoxyribonucleic acid sequence in *Pseudomonas aeruginosa* which mediates resistance to gentamicin and four other antimicrobial agents. J. Bacteriol. **139:**877–882.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
34. **Scavizzi, M. R.** 1973. Nouveaux groupes d'incompatibilité des plasmides. Intérêt dans les épidémies de crèches à Escherichia coli O111:B4. Ann. Microbiol. (Institut Pasteur) **124B:**153–167.
35. **Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller.** 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Rev. **57:**138–163.
36. **Simonsen, C. C., E. Y. Chen, and A. D. Levinson.** 1983. Identification of the type I trimethoprim-resistant dihydrofolate reductase specified by the *Escherichia coli* R-plasmid R483: comparison with procaryotic and eucaryotic dihydrofolate reductases. J. Bacteriol. **155:**1001–1008.
37. **Stokes, H. W., and R. M. Hall.** 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol. Microbiol. **3:**1669–1683.
38. **Stokes, H. W., and R. M. Hall.** 1992. The integron In1 in plasmid R46 includes two copies of the *oxa2* gene cassette. Plasmid **28:**225–234.
39. **Sundström, L., P. Rådström, G. Swedberg, and O. Sköld.** 1988. Site-specific recombination promotes linkage between trimethoprim- and sulfonamide resistance genes. Sequence characterization of *dhfrV* and *sulI* and a recombination active locus of Tn*21*. Mol. Gen. Genet. **213:**191–201.
40. **Sundström, L., P. H. Roy, and O. Sköld.** 1991. Site-specific insertion of three structural gene cassettes in transposon Tn*7*. J. Bacteriol. **173:**3025–3028.
41. **Sundström, L., and O. Sköld.** 1990. The *dhfrI* trimethoprim resistance gene of Tn*7* can be found at specific sites in other genetic surroundings. Antimicrob. Agents Chemother. **34:**642–650.
42. **Sutcliffe, J. G.** 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43:**77–90.
43. **Tait, R. C., H. Rempel, R. L. Rodriguez, and C. I. Kado.** 1985. The aminoglycoside-resistance operon of the plasmid pSa: nucleotide sequence of the streptomycin-spectinomycin resistance gene. Gene **36:**97–104.
44. **Tenover, F. C., D. Filpula, K. L. Phillips, and J. J. Plorde.** 1988. Cloning and sequencing of a gene encoding an aminoglycoside 6′-*N*-acetyltransferase from an R factor of *Citrobacter diversus*. J. Bacteriol. **170:**471–473.
45. **Tolmasky, M. E.** 1990. Sequencing and expression of *aadA*, *bla*, and *tnpR* from the multiresistance transposon Tn*1331*. Plasmid **24:**218–226.
46. **Tolmasky, M. E., and J. H. Crosa.** 1993. Genetic organization of antibiotic resistance genes (*aac*(*6′*)-*Ib*, *aadA*, and *oxa9*) in the multiresistance transposon Tn*1331*. Plasmid **29:**31–40.
47. **Tran Van Nhieu, G., and E. Collatz.** 1987. Primary structure of an aminoglycoside 6′-*N*-acetyltransferase, AAC(6′)-4, fused in vivo with the signal peptide of the Tn*3*-encoded β-lactamase. J. Bacteriol. **169:**5708–5714.
48. **Vliegenthart, J. S., P. A. G. Ketelaar-van Gaalen, and J. A. M. van de Klundert.** 1989. Nucleotide sequence of the *aacC2* gene, a gentamicin resistance determinant involved in a hospital epidemic of multiply resistant members of the family *Enterobacteriaceae*. Antimicrob. Agents Chemother. **33:**1153–1159.
49. **Wiedemann, B., C. Kliebe, and M. Kresken.** 1989. The epidemiology of β-lactamases. J. Antimicrob. Chemother. **24:**1–22.
50. **Wohlleben, W., W. Arnold, L. Bissonnette, A. Pelletier, A. Tanguay, P. H. Roy, G. C. Gamboa, G. F. Barry, E. Aubert, J. Davies, and S. A. Kagan.** 1989. On the evolution of Tn*21*-like multiresistance transposons: sequence analysis of the gene (*aacC1*) for gentamicin acetyltransferase-3-I (AAC(3)-I), another member of the Tn*21*-based expression cassette. Mol. Gen. Genet. **217:**202–208.
51. **Yamamoto, T., M. Tanaka, R. Baba, and S. Yamagishi.** 1981. Physical and functional mapping of Tn*2603*, a transposon encoding ampicillin, streptomycin, sulfonamide, and mercury resistance. Mol. Gen. Genet. **181:**464–469.