ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1997, p. 1024–1032 Vol. 41, No. 5
0066-4804/97/$04.00+0

Mapping the Regions Carrying the Three Contiguous Antibiotic Resistance Genes *aadE*, *sat4*, and *aphA-3* in the Genomes of Staphylococci

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Received 19 December 1996/Returned for modification 30 January 1997/Accepted 4 March 1997

Tn*5405* (12 kb) is a staphylococcal composite transposon delimited by two inverted copies of IS*1182*, one of which contains IS*1181*. The internal part of this transposon carries three antibiotic resistance genes, *aphA-3*, *aadE*, and *sat4*, and three open reading frames (ORFs), *orfx*, *orfy*, and *orfz*, of unknown function. The dispersion of Tn*5405* and the genes and ORFs included in this transposon were investigated in 50 epidemiologically unrelated staphylococci carrying *aphA-3*. Twenty-three maps, distinguishable by the presence or absence of the investigated genes or ORFs and/or by the sizes of the restriction fragments carrying them, were identified. Four isolates carried Tn*5405*, and 15 other isolates contained a Tn*5405*-related element. IS*1182* was not detected in the *aphA-3* regions mapped in 31 isolates which carried the following combinations: *orfx*, *orfy*, *aadE*, *sat4*, and *aphA-3* ± *orfz*; *orfy*, *aadE*, *sat4*, and *aphA-3* ± *orfz*; and *aadE*, *sat4*, *aphA-3*, and *orfz*. In all isolates, the genes and ORFs investigated were in relative positions similar to those in Tn*5405*. Thus, the internal part of Tn*5405* appeared to be partially conserved with the maintenance, in all of the isolates, of at least the three antibiotic resistance genes.

We have previously characterized a 12-kb composite transposon, Tn*5405* (8), from the chromosome of a methicillin-resistant *Staphylococcus aureus* (MRSA) isolate, BM3121 (9). This transposon is delimited by two inverted copies of the insertion sequence IS*1182* (8), and one of these copies contains another insertion sequence, IS*1181* (6). Tn*5405* carries the genes *aphA-3* (12) and *aadE* (11), which encode resistance to aminoglycosides modified by APH(3′)-III (kanamycin, lividomycin, neomycin, and amikacin) and AAD(6′) (streptomycin), respectively. It also carries the gene *sat4* (15), which encodes resistance to streptothricin and three open reading frames (ORFs), *orfx*, *orfy*, and *orfz*, which encode putative proteins of unknown biological function (5). The three antibiotic resistance genes carried by Tn*5405* were mapped to a 3.2-kb *Hin*dIII fragment also detected in an *S. aureus* plasmid, pIP1718, which does not contain nucleotide sequences hybridizing with IS*1182* or IS*1181* (5). Udo and Grubb (24) have described a 4.5-kb transposable element, Tn*3854*, conferring resistance to kanamycin, neomycin, and streptomycin. The map and the nucleotide sequence of Tn*3854*, which probably carries *aphA-3* and *aadE*, are not available. Nevertheless, these observations suggest that Tn*5405* is not the only putative mobile element in staphylococci carrying *aphA-3* and *aadE*. We evaluated the distribution of Tn*5405* in 50 independent staphylococcus isolates harboring *aphA-3*. We also tested the *aphA-3* regions of the genomes of isolates which do not contain Tn*5405* for the presence of genes or ORFs detected in this transposon, so as to identify other putative mobile elements which may also contribute to the dissemination of *aphA-3*.

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MATERIALS AND METHODS

Bacteria and plasmids. Fifty staphylococcal isolates of various species, collected between 1978 and 1995 inclusive, were studied (Table 1). They were isolated from epidemiologically unrelated hospitalized patients in various geographical areas. They were chosen as having DNA hybridizing with an *aphA-3* probe. The phenotypic tests used to identify the isolates have been previously described (4) and included ID32 Staph System (BioMérieux, Marcy-l'Etoile, France).

Plasmid pIP1718 from *S. aureus* BM3505 (5) was used as a template for sequencing, and the *Escherichia coli* plasmid pUC18 (25) was used as a cloning vector. Plasmid pIP1644 (18) is pUC18 carrying, between its *Pst*I and *Sma*I sites, a 615-bp fragment amplified from the transposase-encoding gene of IS*257* by using a primer, Pɪs*257*a, modified to include a *Pst*I site and Pɪs*257*b (Table 2).

Media. Staphylococci were grown in brain heart infusion (Difco Laboratories, Detroit, Mich.), and *E. coli* cells were grown in Luria broth (LB) (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl/liter). Solid media contained 15 g of agar/liter. Bacteria were incubated at 37°C and for liquid cultures with agitation. Susceptibility to antibiotics was tested on Mueller-Hinton agar (MHA) (Diagnostics Pasteur, Marne-la-Coquette, France).

Susceptibility to antibiotics. Susceptibility to antibiotics was determined by a disk diffusion assay (2) with commercially available antibiotic disks (Diagnostic Pasteur) and streptothricin (30 μg) disks prepared in our laboratory. MICs of streptothricin were determined with serial twofold dilutions of antibiotics in MHA (10).

DNA isolation and analysis. Total cellular DNA and plasmid DNA were isolated from staphylococcal isolates and were purified as described previously (3, 7). Plasmid DNA was isolated from *E. coli* by a rapid alkaline extraction procedure (1). Cesium chloride density gradient centrifugation of cleared lysates (19) was used for large-scale plasmid preparations.

Restriction endonucleases were obtained from Amersham International (Buckinghamshire, United Kingdom) or from Pharmacia (Uppsala, Sweden) and were used according to the manufacturer's instructions. Total cellular DNA, plasmids, PCR, and restriction fragments were analyzed by 0.7 to 1.5% agarose gel electrophoresis in Tris-acetate buffer (40 mM Tris-acetate, 1 mM EDTA) as described by Sambrook et al. (19). Before cleavage with the appropriate endonucleases, PCR fragments were purified with microcon 100 (Amicon, Epernon, France). The Raoul I ladder (Appligène, Strasbourg, France) and 1-kb DNA ladder (Bethesda Research Laboratories, Bethesda, Md.) were used as molecular weight markers. *Sma*I digestion and pulsed-field gel electrophoresis (PFGE) were performed as described previously (8).

PCR. DNA was amplified by PCR as previously described (8). *Taq* DNA polymerase (Amersham International) was used for amplification of fragments with sizes of <2 kb, and High *Taq* DNA polymerase (Bioprobe, Montreuil, France) was used for amplification of fragments with sizes of >2 kb. The time and temperature profiles of PCR experiments differed, depending on the size of

TABLE 1. Relevant characteristics of the 50 staphylococcal isolates tested

Map (cf. Fig.2)	Isolate designation	Species (yr of sampling)	Relevant antibiotic resistance markers[a]	Size (kb) of PCR fragments amplified with primers[b]:					Size (kb) of *Bgl*II fragment hybridizing with *orfz*[c]
				Psat1/Psat2	Ps1/Pn2	Ps4/Pn3	Pn1/Pz2	Pn1/Pz3	
I	BM3121	*S. aureus* (1979)	Mc Nm Sm						
	BM3125	*S. aureus* (1978)	Mc Nm Sm						
	BM3318	*S. aureus* (1983)	Mc Nm Sm	0.48	2.2	ND	2.5	ND	11.5
	BM3474	*S. epidermidis* (1984)	Nm Sm						
II	BM3176	*S. aureus* (1978)	Mc Nm Sm St						
	BM3182	*S. aureus* (1978)	Mc Nm Sm St						
	BM3247	*S. aureus* (1983)	Mc Nm Sm St						
	BM3396	*S. aureus* (1984)	Nm Sm						
	BM3623	*S. aureus* (1985)	Mc Nm Sm St						
	BM3647	*S. aureus* (1985)	Mc Nm Sm						
	BM3649	*S. aureus* (1985)	Mc Nm Sm St	0.48	2.2	ND	2.5	ND	10
	BM3670	*S. aureus* (1984)	Mc Nm Sm St						
	BM3684	*S. haemolyticus* (1985)	Mc Sm						
	BM3686	*S. saprophyticus* (1985)	Mc Sm						
	BM9020	*S. epidermidis* (1985)	Mc Sm						
	BM9056	*S. aureus* (1984)	Mc Nm Sm St						
IIIa	BM10842	*S. aureus* (1993)	Nm Sm	–	1.8				
IIIb	BM3472	*S. aureus* (1985)	Nm Sm	–	2.0	ND	ND	ND	–
IIIb	BM9015	*S. aureus* (1985)	Nm Sm	–	2.0				
IV	BM3505	*S. aureus* (1985)	Mc Nm Sm St	0.48	2.2	ND	–	ND	ND[d]
V	BM3698	*S. epidermidis* (1985)	Mn Nm Sm St	0.48	2.2	ND	–	–	5
	BM10173	*S. epidermidis* (1986)	Mc Nm Sm St						
VI	BM9895	*S. epidermidis* (1989)	Mc Nm Sm St	0.48	2.2	ND	–	–	5.4
VII	BM12071	*S. aureus* (1995)	Nm Sm St	0.48	2.2	ND	–	–	11.5
VIII	BM10819	*S. haemolyticus* (1994)	Mc Nm Sm St	0.48	2.2	ND	–	–	–
IX	BM3235	*S. aureus* (1983)	Mc Nm Sm	0.48	2.2	ND	2.5	ND	>50
X	BM3642	*S. aureus* (1985)	Mc Nm Sm	0.48	2.0	–	–	ND	–
XI	BM12050	*S. aureus* (1995)	Nm Sm St	0.48	2.2	ND	–	ND	–
XIIa	BM9451	*S. epidermidis* (1987)	Mc Nm Sm	0.6	–	0.9	–	2	>50
XIIb	BM9454	*S. epidermidis* (1987)	Mc Nm Sm						
XIII	BM9443	*S. epidermidis* (1987)	Mc Nm Sm	0.6	–	0.9	–	–	>50
XIV	BM10164	*S. epidermidis* (1983)	Nm	0.48	–	0.8	2.5	ND	7
XV	BM9002	*S. haemolyticus* (1985)	Mc Nm Sm	0.48	2.2	0.8	2.5	ND	>50
XVI	BM3588	*S. haemolyticus* (1985)	Mc Sm	0.48	–	0.8	2.5	ND	>50
XVII	BM3683	*S. warneri* (1985)	Mc Sm	0.6	–	0.9	2.5	ND	>50
XVIII	BM3689	*S. haemolyticus* (1985)	Nm Sm						
	BM9001	*S. epidermidis* (1985)	Mc Nm Sm						
	BM9436	*S. epidermidis* (1987)	Mc Nm Sm	0.6	–	0.9	–	2	>50
	BM9448	*S. epidermidis* (1987)	Mc Nm Sm						
	BM12023	*S. epidermidis* (1994)	Mc Nm						
	BM9017	*S. epidermidis* (1985)	Mc Nm Sm						
	BM9450	*S. epidermidis* (1987)	Mc Nm						
XIX	BM9461	*S. epidermidis* (1987)	Mc Nm Sm	0.6	–	0.9	–	2	>50
	BM9462	*S. epidermidis* (1987)	Mc Nm Sm						
	BM9880	*S. haemolyticus* (1989)	Mc Nm Sm						
XX	BM3681	*S. haemolyticus* (1985)	Mc Nm Sm	0.6	–	0.9	–	–	>50
	BM3685	*S. haemolyticus* (1985)	Mc Nm Sm						
XXI	BM9879	*S. haemolyticus* (1989)	Mc Nm Sm	0.6	–	0.9	–	2	>50
XXII	BM9437	*S. haemolyticus* (1987)	Mc Nm Sm	0.7	–	1.0	2.5	ND	>50
XXIII	BM3691	*S. haemolyticus* (1985)	Mc Nm Sm	0.7	–	1.0	2.5	ND	>50

[a] Only resistance to methicillin (Mc), neomycin (Nm), streptomycin (Sm), and streptothricin (St) is reported.

[b] Only relevant data obtained with some pairs of primers (Fig. 1) are given. Further data are reported in Fig. 2. PCR experiments were not done (ND) for isolates in which the ORFs and genes were not found by hybridization experiments or because they could be mapped with the other pairs of primers reported (Fig. 2). A minus sign indicates there was no amplification product with a given pair of primers.

[c] In each of the 43 isolates hybridizing with *orfz*, *orfz* was carried by a *Bgl*II fragment of the same size as that hybridizing with *aphA-3*. However, the use of the two pairs of primers Pn1/Pz2 and Pn1/Pz3 did not enable us to locate *orfz* in maps V to VII, XIII, and XX (Fig. 2).

[d] The absence of *orfz* in map IV (Fig. 2) was demonstrated by analysis of the nucleotide sequence between the end of *aphA-3* and the left copy of IS*257*.

TABLE 2. Oligonucleotides used in PCR experiments in this study

Oligonucleotide	Primer sequence[a]	Nucleotide position on the published sequence	Reference
P1	5′-CCCGATGAAGTCTTTCTTCC-3′	1151–1170 in IS*1182*	8
F	5′-GGCGGCCAGTCCATTATTGGGC-3′	1766–1745 in IS*1181*	6
Pn1	5′-GCTGCGTAAAAGATACGGAAGG-3′	345–366 in *aphA-3*	12
Pn2	5′-CCCAATCAGGCTTGATCCCC-3′	1043–1024 in *aphA-3*	12
Pn3	5′-CCTTCCGTATCTTTTACGCAGC-3′	366–345 in *aphA-3*	12
Pn4	5′-GGGGATCAAGCCTGATTGGG-3′	1024–1043 in *aphA-3*	12
Ps1	5′-GCAGAACAGGATGAACGTATTCG-3′	1132–1154 in *aadE*	11
Ps2	5′-CGGCATATGTGCTATCCAGGC-3′	1969–1949 in *aadE*	11
Ps3	5′-CGAATACGTTCATCCTGTTCTGC-3′	1154–1132 in *aadE*	11
Ps4	5′-GCCTGGATAGCACATATGCCG-3′	1949–1969 in *aadE*	11
Py1	5′-GCTCGCAGAAAGGACTGGCTGG-3′	381–402 in *orfy*	5
Py2	5′-GGATGGCGGATGAAATGCGACG-3′	996–1017 in *orfy*	5
Px1	5′-GCACCCATACAGAGGATTCGG-3′	2328–2348 in *orfx*	5
Px2	5′-GCTTCTCGGTCATGTCGTAGC-3′	3023–3003 in *orfx*	5
Px3	5′-CCTGAAAAATATACCGAGAAGG-3′	2939–2960 in *orfx*	5
P*sat*1	5′-GCAGAGCACCTGAAAGATATCG-3′	392–413 in *sat4*	15
P*sat*2	5′-GCGTATAACATAGTATCGACGG-3′	864–843 in *sat4*	15
Pz1	5′-GCACACACAAATTAGCCTTGATGG-3′	7485–7508 in *orfz*	5
Pz2	5′-CCACCGCCACTGTCTGTCCGC-3′	7924–7904 in *orfz*	5
Pz3	5′-CCATCAAGGCTAATTTGTGTGTGC-3′	7508–7485 in *orfz*	5
P*IS257*a	5′-GTTATCA**CTGCAG**CCGTTGG-3′	93–112 in IS*257*	18
P*IS257*b	5′-CGTACTACGATTAAAGCACC-3′	720–701 in IS*257*	18
P*IS257*c	5′-GCCAACGGCTACAGTGATAACATCC-3′	113–89 in IS*257*	18
P*IS257*d	5′-GGTCTCTTCAGATCTACGG-3′	670–688 in IS*257*	18

[a] Underlined boldface section represents a *Pst*I site.

the expected PCR fragments. For amplification of fragments <2 kb in size, the program was 5 min at 95°C, 2 min at 60°C, and then 30 cycles at 72°C (1 min), 95°C (45 s), and 60°C (1 min). For amplification of fragments >2 kb in size, the program was 5 min at 95°C, 2 min at 60°C, and then 30 cycles at 72°C (1 min 30 s), 95°C (45 s), and 60°C (1 min).

The oligonucleotides were selected to correspond to sequences in IS*1182* (8), IS*1181* (6), *aphA-3* (12), *aadE* (11), *sat4* (15), *orfx* (5), *orfy* (5), *orfz* (5), and IS*257* (18), as shown in Table 2.

Blotting and hybridization. DNA was transferred from agarose gels to Hybond-N^+ membranes (Amersham International) by the capillary blotting method (4). Prehybridization and hybridization were performed under stringent conditions as previously described (8). Amplification products used as probes were electrophoresed in agarose gels, extracted from the gel, and purified with the Gene Clean II kit (Bio 101, La Jolla, Calif.). The amplified fragments and plasmid pIP1644 were labeled with [α-^{32}P]dCTP (110 TBq · mmol^{-1}) by random priming with the Megaprime DNA labeling system (Amersham International). The blots were exposed to Hyperfilm (Amersham International) at −80°C.

Cloning and DNA sequencing. Standard methods were used for DNA cloning (19). *E. coli* TG1 was transformed by the method of Hanahan (13), and transformants were selected on ampicillin (100 μg/ml)-LB-agar.

DNA was sequenced by the dideoxynucleotide chain termination method (20) with [α-^{35}S]dATP (Amersham International) for labeling, with a template consisting of double-stranded plasmid DNA. The 17-nucleotide universal primers (Pharmacia) and the T7 polymerase sequencing kit (Pharmacia) were used according to the supplier's instructions.

RESULTS

aphA-3 regions were mapped in 50 epidemiologically unrelated staphylococcal isolates (Table 1). The *Sma*I restriction patterns of the MRSA isolates which differed by more than three bands suggested that they belong to distinct genotypes (results not shown). The other isolates included in this study had distinct antibiotypes, and those which had extrachromosomal DNA bands as assessed by electrophoresis of total cellular DNA in agarose gels carried different plasmids (results not shown). Cellular DNA of each of the isolates was cleaved by *Bgl*II, *Cla*I, *Hin*dIII, and *Eco*RI separately and hybridized with DNA fragments of *aphA-3* (12), *aadE* (11), *sat4* (15), *orfx* (5), *orfy* (5), *orfz* (5), IS*1181* (6), IS*1182* (8), and IS*257* (18). The map of Tn*5405* is shown in Fig. 1. The pairs of primers used in PCR experiments to map and to determine the orientations of the genes, ORFs, and insertion sequences carried by Tn*5405* are indicated in Fig. 1 and 2 and in Table 2. Different roman numerals were assigned to maps distinguishable by either the presence or the absence of any of the insertion sequences, genes, or ORFs carried by Tn*5405* or by the size of any of the *Bgl*II, *Cla*I, or *Hin*dIII fragments hybridizing with the *aphA-3* probe. Twenty-three different maps (I to XXIII) were identified among the 50 isolates tested. For maps with the same roman numeral but differing by the size of a fragment amplified with a given pair of primers, letter suffixes were added. Thus maps III and XII were subdivided into two categories, a and b (Fig. 2 and Table 1).

The 50 staphylococcal isolates hybridized with the *aphA-3*, *aadE*, and *sat4* probes. In each isolate, the nucleotide sequences hybridizing with the three probes were all detected on a single *Hin*dIII fragment. The sizes of this fragment varied among the isolates from 2.8 to 3.4 kb (Fig. 2). In some cases, the *Hin*dIII fragments carrying *aphA-3* included *orfy* and part of *orfx*. The sizes of the amplicons obtained with the primer pairs Ps1/Pn2 (1.8 to 2 to 2.2 kb) or Ps4/Pn3 (0.8 to 0.9 to 1 kb) (Fig. 2 and Table 1) suggest that the genes *aadE*, *sat4*, and *aphA-3* have the same relative positions in all of the isolates. The difference between the sizes of these amplicons appeared to be due, in most cases, to rearrangements in the *sat4* gene. Primers Psat1/Psat2 either did not amplify a fragment (maps IIIa and IIIb) or amplified intra-*sat4* amplicons of various sizes: 0.48, 0.6, or 0.7 kb (Fig. 2 and Table 1). In 28 of the 50 isolates, the intra-*sat4* amplicon was 0.48 kb. Only 14 of these isolates were resistant to streptothricin (Table 1), suggesting that many isolates carried sequence modifications undetectable by size analysis of the intra-*sat4* amplicons but sufficient to abolish expression. The 22 isolates for which the intra-*sat4* amplicons were 0.6 or 0.7 kb were all susceptible to streptothricin (Table 1). Despite the detection of an amplicon having the same size as that in Tn*5405* with primers Ps1/Pn2 (2.2 kb [Fig. 1]), resistance to aminoglycosides was not necessarily

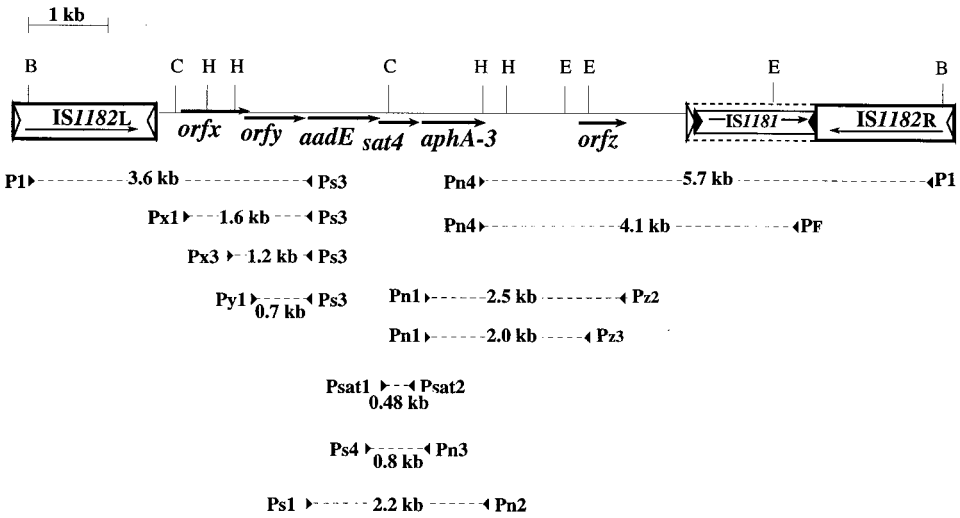

FIG. 1. Physical map of Tn*5405*. The primers used to detect, locate, and orient the genes and ORFs of Tn*5405* in the staphylococcal isolates tested in this study are indicated below the map. The sizes of the amplicons obtained with the isolate BM3121 carrying Tn*5405* are also reported. The nucleotide sequence of the 6.9-kb region of Tn*5405* delimited by the two inverted copies of IS*1182* has been submitted to GenBank under the following accession no.: U73025 for the left region flanked by IS*1182*L and *orfy* (nucleotides [nt] 1832 to 3195), U73026 for the intergenic region flanked by *aadE* and *aphA-3* (nt 4558 to 5365), and U73027 for the right region flanked by *aphA-3* and IS*1182*R (nt 6102 to 8784). B, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII.

expressed (Table 1), whereas the *aphA-3* and *aadE* genes of Tn*5405* carried by BM3121 and isolated by transduction (8, 9) confer resistance to the aminoglycosides modified by APH(3′)-III and AAD(6′).

In three isolates, BM3698, BM9895, and BM12071 (Table 1, maps V, VI, and VII, respectively), the nucleotide sequences hybridizing with the *aadE*, *sat4*, and *aphA-3* probes were carried by bands migrating above the chromosomal DNA (data not shown), suggesting that they are located on a plasmid. BM3505 (Table 1, map IV) has been shown to harbor a plasmid, pIP1718, carrying *aadE*, *sat4*, and *aphA-3* (5). This plasmid is at least 25 kb long, since cleavage with *Sma*I generates a linear 25-kb fragment hybridizing with the *aphA-3* probe (data not shown). In all of the other isolates, the hybridization signal, after 0.7% agarose gel electrophoresis of the total DNA content, appeared to be in the chromosomal DNA (data not shown). The sizes of the *Sma*I fragments hybridizing with the *aphA-3*-probe in six of the isolates were determined by PFGE: 673 kb for isolates BM3121 (Table 1, map I), BM3125 (Table 1, map I), and BM3247 (Table 1, map II); 370 kb for BM9056 (Table 1, map II); 360 kb for BM3623 (Table 1, map II); and 324 kb for BM3176 (Table 1, map II) (data not shown).

A structure indistinguishable from that of Tn*5405* (Fig. 1) was found in only 4 of the 50 isolates tested: 3 MRSA isolates and 1 *S. epidermidis* isolate, all isolated between 1978 and 1984 (Fig. 2 and Table 1, map I). Fifteen other *Staphylococcus* isolates (12 *S. aureus* isolates, 1 *S. epidermidis* isolate, 1 *S. haemolyticus* isolate, and 1 *S. saprophyticus* isolate) collected between 1978 and 1993 harbored elements delimited by two inverted copies of IS*1182* and containing *orfx*, *orfy*, *aadE*, *sat4*, and *aphA-3* ± *orfz* in relative positions similar to those in Tn*5405* (Fig. 2 and Table 1, maps II, IIIa, and IIIb). Map II differed from map I by the absence of IS*1181* in IS*1182*R of Tn*5405*. Maps IIIa and IIIb do not contain IS*1181* and *orfz*, and the size of the *Hin*dIII fragment carrying *orfy*, *aadE*, *sat4*, and *aphA-3* is slightly larger (3.4 kb) than that in maps I and II (3.2 kb). Maps IIIa and IIIb differ by the sizes of the amplicons obtained with primers Ps1/Pn2 (1.8 and 2 kb, respectively), which in both cases are smaller than those of maps I and II (2.2 kb). Nucleotide sequences hybridizing with IS*1182* were not detected in the 31 *S. aureus* and coagulase-negative isolates having maps IV to XXIII, which were isolated between 1983 and 1995.

In each of maps IV to XXIII, the *Bgl*II fragment carrying the combination of *aadE*, *sat4*, and *aphA-3* is either 4.8, 5, 5.4, 7, 11.5, or >50 kb in size (Fig. 2). The only known staphylococcal insertion sequences containing a single *Bgl*II site are IS*1182* (8) and IS*257* (18). Because the isolates with maps IV to XXIII do not contain IS*1182*, the presence of IS*257* was suspected. The cellular DNA of these isolates was tested for hybridization with the IS*257* probe, pIP1644. Most of the isolates carried multiple copies of IS*257*. PCR experiments were carried out to map and determine the orientation of IS*257* in the region of interest. Eight pairs of primers were selected (PIS*257*a/Ps3, PIS*257*b/Ps3, PIS*257*c/Ps3, PIS*257*d/Ps3, PIS*257*a/Pn4, PIS*257*b/Pn4, PIS*257*c/Pn4, and PIS*257*d/Pn4) to analyze the isolates having maps IV to VII and XIV in which the *Bgl*II fragments carrying *aphA-3* were between 4.8 and 11.5 kb in size. Isolates with maps VIII to XIII and XV to XXIII were not tested because the *Bgl*II fragments carrying *aphA-3* were large (>50 kb). No amplification products were detected in the isolates having maps VII and XIV. Fragments with sizes of 1.4 or 1.8 kb were amplified from DNA with maps IV, V, and VI with primers PIS*257*a/Pn4 (Fig. 2), suggesting the presence of a copy of IS*257* near *aphA-3*. No amplification product was obtained with PIS*257*a/Ps3, PIS*257*b/Ps3, or PIS*257*c/Ps3. Primers PIS*257*d/Ps3 gave an amplicon with a size of 1.6 kb only with plasmid pIP1718 harbored by BM3505 (Fig. 2 and Table 1, map IV). These results suggest the presence of an incomplete copy of IS*257* at the left part of map IV and the absence of IS*257* at the left part of maps V and VI. There is a *Cla*I site 600 bp upstream from the *Bgl*II site of IS*257* in map IV, and because IS*257* contains no *Cla*I site, the insertion sequence was presumably incomplete. The 4.8-kb *Bgl*II fragment of pIP1718 was inserted into the *Bam*HI site of pUC18, and the ends of the fragment were sequenced (150 nucleotides on the left and 170 nucleotides on the right). The data (not shown) confirmed the presence of IS*257* nucleotide sequences at both ends and indicated that *orfx* is interrupted by IS*257* nucleotide sequences (Fig. 2, map IV).

The isolates carrying *orfx*, *orfy*, *aadE*, *sat4*, and *aphA-3* ± *orfz*

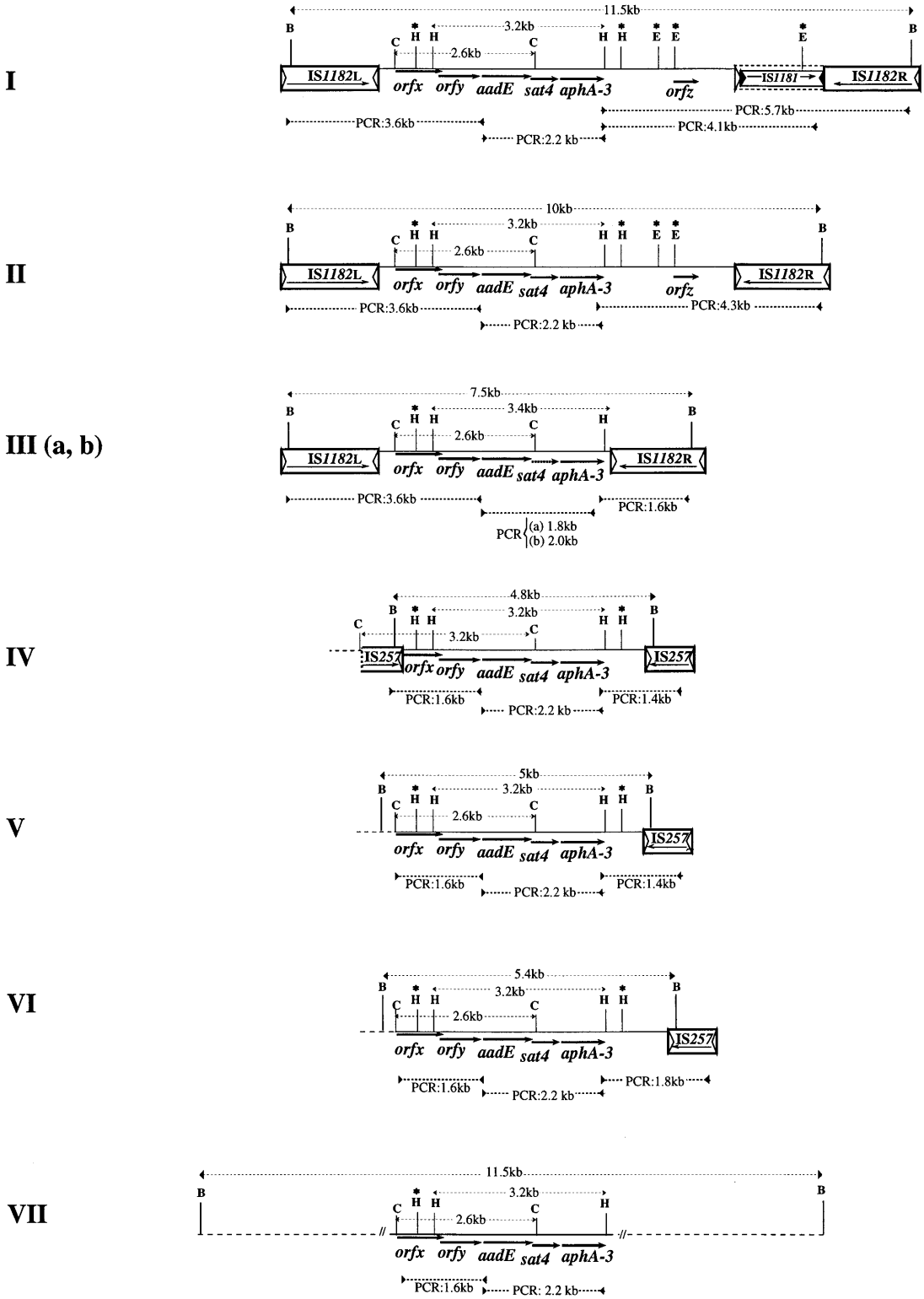

FIG. 2. Maps of the regions carrying *aphA-3* for 50 independent staphylococci (Table 1). The 23 roman numerals were assigned to the maps distinguishable by either the presence or the absence of any of the insertion sequences, genes or ORFs carried by Tn*5405* or by the size of any of the *Bgl*II, *Cla*I, or *Hin*dIII fragments hybridizing with the *aphA-3* probe. For maps of the same roman numeral differing by the size of a fragment amplified with a given pair of primers, letter suffixes are added. The *Hin*dIII and *Eco*RI sites indicated by asterisks were mapped by digestion of the PCR-amplified fragments reported below the maps.

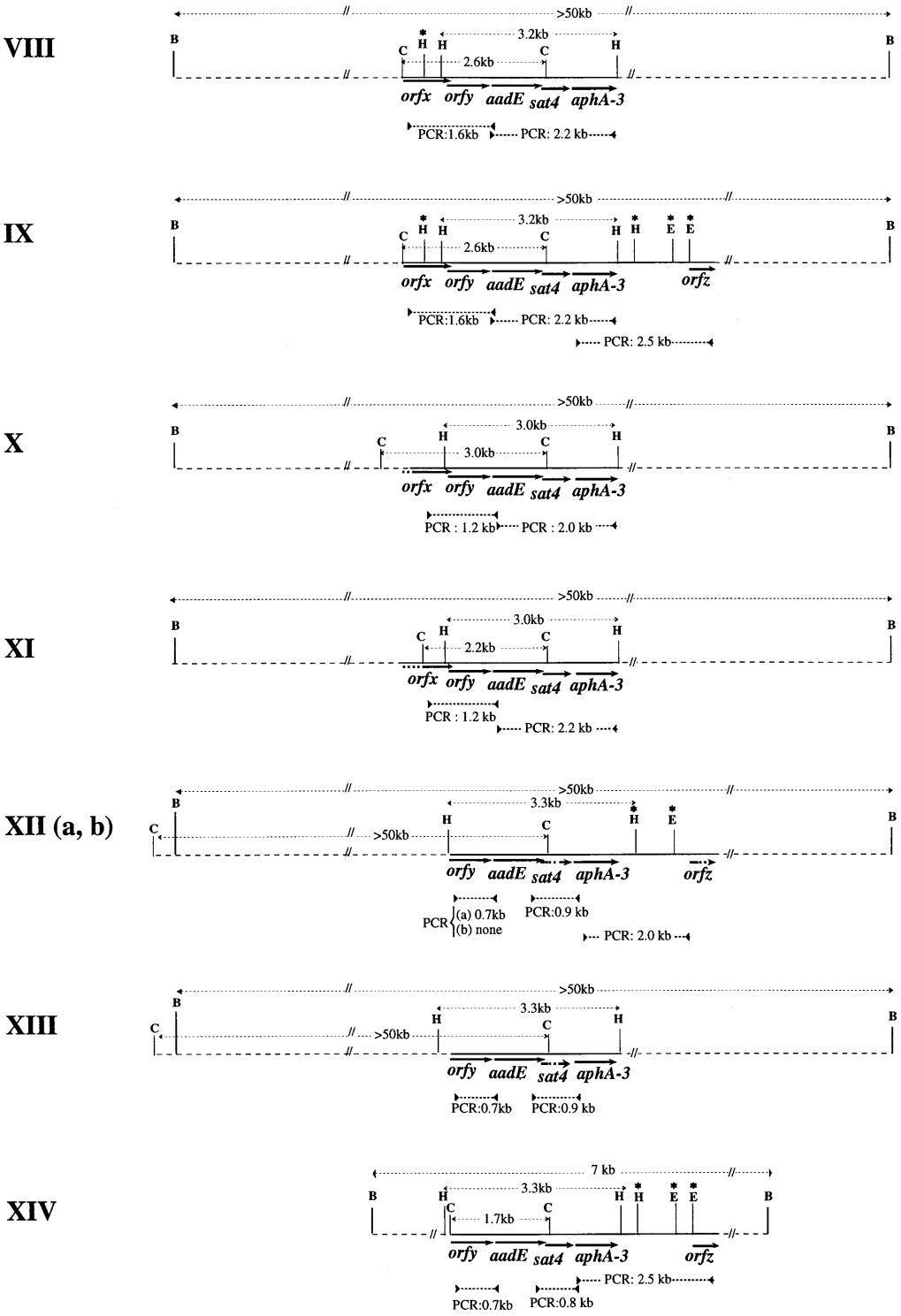

FIG. 2—*Continued.*

(maps I to XI) were *S. aureus* or coagulase-negative species. Those lacking *orfx* (maps XII to XIV) or *orfx* and *orfy* (maps XV to XXIII) were all coagulase-negative species. Forty-three of the 50 isolates contained nucleotide sequences hybridizing with the *orfz* probe (Table 1). In each of the 43 isolates, *orfz* was carried by a *Bgl*II fragment of the same size as that hybridizing with *aphA-3*. Despite the use of two pairs of primers, Pn1/Pz2 and Pn1/Pz3 (Fig. 1), *orfz* could not be located in maps V to VII, XIII, and XX. The absence of amplification with these primers may occur either because they carry *orfz*-like copies which do not hybridize with primers Pz2 or Pz3 or because the *orfz* gene is distant and is not necessarily downstream from *aphA-3* or is on a *Bgl*II fragment distinct from that containing *aphA-3*, despite its similar size.

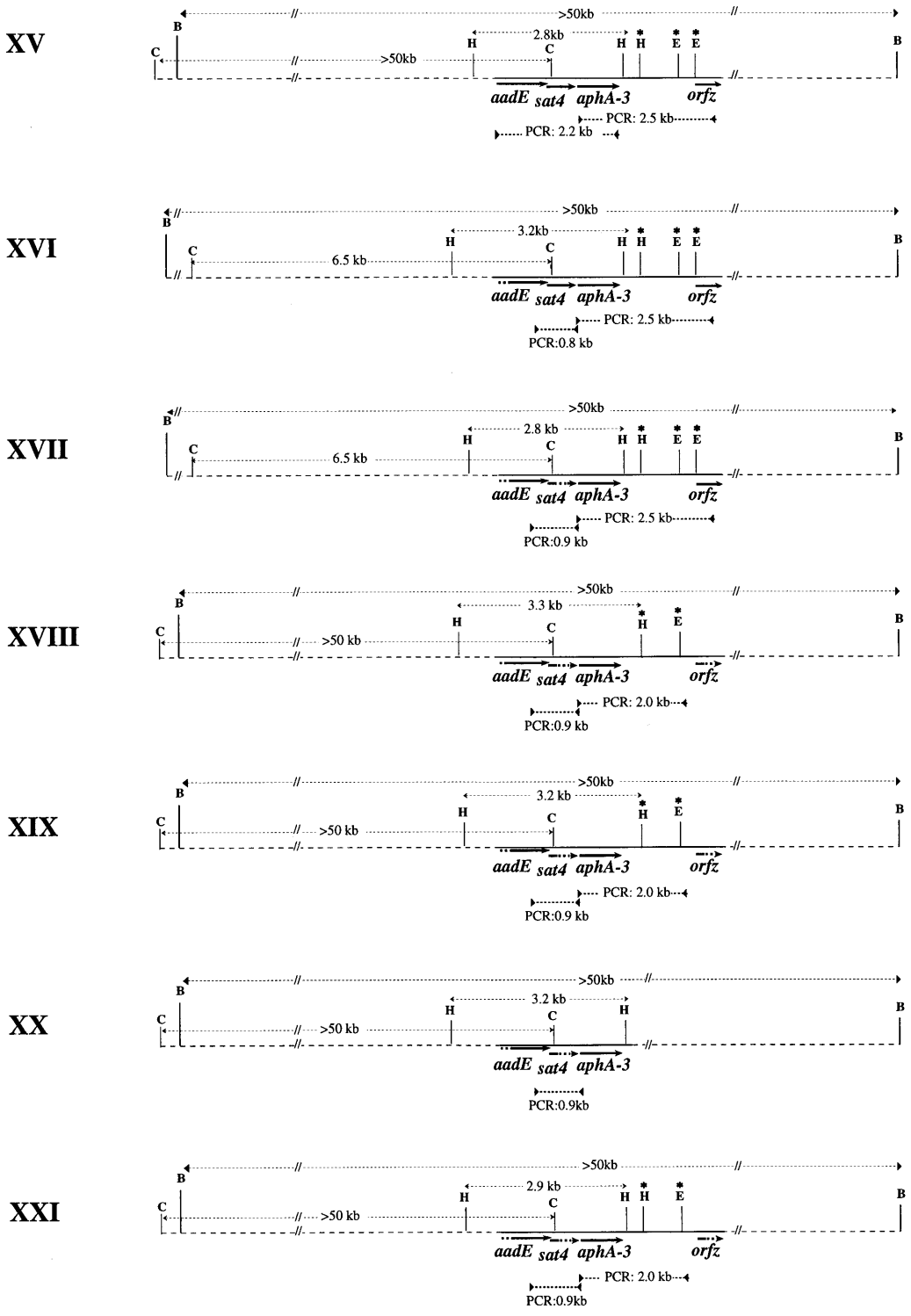

FIG. 2—*Continued.*

DISCUSSION

Staphylococci carry a relatively well-conserved region containing the three antibiotic resistance genes found in Tn*5405* (*aadE*, *sat4*, and *aphA-3*) (5, 8). However, the structures of this region in the genome differed substantially among isolates. This heterogeneity may be due to structural instability or to the common ancestor carrying *aadE*, *sat4*, and *aphA-3* being ancient enough in staphylococci to explain the multiplicity of rearrangements. The first staphylococci resistant to the aminoglycosides modified by the enzymes APH-3′-III and AAD6′ were described in 1959 (17). In contrast, all of the tested

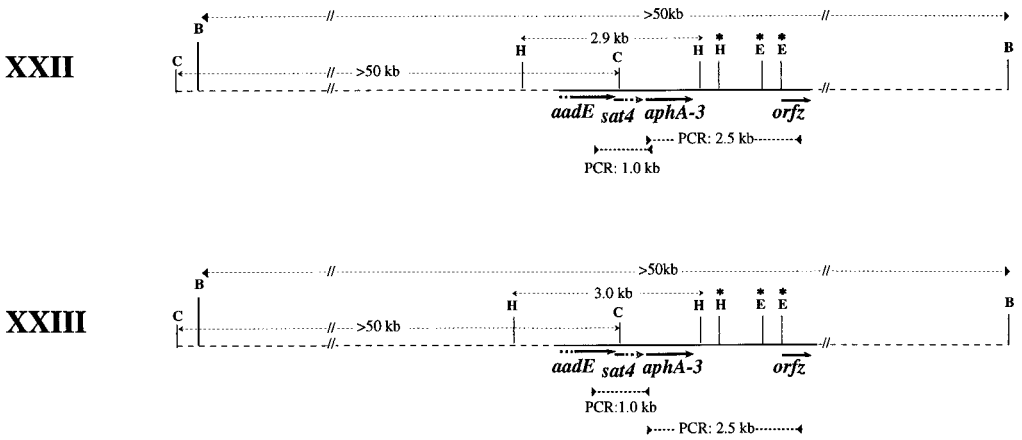

FIG. 2—*Continued.*

staphylococci resistant to the aminoglycosides modified by the bifunctional enzyme AAC(6′)-APH(2″) and which were selected more recently (1975) (17) carry closely related transposons, Tn*4001* (16) or Tn*4031* (21), also detected in *Enterococcus faecalis* isolates (Tn*5281*) (14).

The coinheritance of several antibiotic resistance genes will tend to favor the maintenance in the bacteria of each of them, since a selective pressure can be exerted by a single antibiotic. The reason why the three genes *aadE*, *sat4*, and *aphA-3* are regularly associated has not yet been elucidated. It may be because they have a common promoter. The investigation of promoters by sequence analysis is not yet possible in the staphylococcal genome because of the high percentage of adenine and thymine (61 to 70%). Thus, the transcription products have to be analyzed. Alternatively, the three antibiotic resistance genes may be linked simply because they are spread by a mobile element such as a transposon, phage, or plasmid carrying all three genes which has subsequently undergone rearrangements, yielding the maps described in this study.

Parts of the *aadE* and *aphA-3* genes have been detected upstream and downstream, respectively, from the *sat4* gene of *Campylobacter coli* BE/G4 (15). Furthermore, in *C. coli* BM2509 (23), the *aphA-3* gene is preceded by a sequence homologous to part of *sat4* (15) and the promoter of the *aphA-3* gene has been located within this sequence. Part of *orfx* and entire copies of *orfy*, *aadE*, *sat4*, and *aphA-3* are found in the *E. faecalis* plasmid pJH1 (11, 22), in the same relative positions as in Tn*5405* (5, 8). These observations suggest that the three antibiotic resistance genes have been dispersed by horizontal intergeneric transfer of structurally related mobile elements.

Among the isolates carrying *aadE*, *sat4*, and *aphA-3* characterized in this study (Fig. 2), those which may have obtained the genes by insertion of a transposon are delimited by two inverted copies of IS*1182* (maps I, II, and III); they were detected in 19 of the 50 staphylococcal isolates tested (15 *S. aureus* isolates and 4 coagulase-negative staphylococcal isolates). To determine whether such elements have contributed to the intergeneric dispersion of the genes *aadE*, *sat4*, and *aphA-3*, it would be necessary to investigate whether these genes are also flanked by IS*1182* in *C. coli* and *E. faecalis*. Among the staphylococci tested in this study, the smallest element flanked by IS*1182* and including the adjacent ORFs and genes detected in Tn*5405* (*orfx*, *orfy*, *aadE*, *sat4*, and *aphA-3*) is 8 kb long (Fig. 2, map III), which is larger than the 4.5-kb transposon Tn*3854*, which was characterized by Udo and Grubb (24). It is not yet known whether Tn*3854* includes *sat4* or any of the three ORFs carried by Tn*5405*. In isolate BM3121 (Table 1, map I), Tn*5405* is inserted in another transposon, Tn*5404* (7, 8). The presence of direct repeats adjacent to the ends of Tn*5405* suggests that its insertion occurred by transposition. The copy of Tn*5404* carried by BM3121 is functional, since it has been transposed from the chromosome to a plasmid harbored by the isolate (7). However, the ability of the BM3121 copy of Tn*5405* inserted within Tn*5404* to move autonomously is doubtful because of the presence of a stop codon apparently interrupting the transposase gene of both copies of IS*1182* delimiting Tn*5405* (8).

The 31 staphylococcal isolates with maps IV to XXIII do not carry nucleotide sequences hybridizing with IS*1182*, but nucleotide sequences hybridizing with IS*257* were detected in all of these isolates. The four isolates having maps IV, V, and VI (Table 1) had a copy of IS*257* in the vicinity of *aadE*, *sat4*, and *aphA-3* (Fig. 2). The presence of IS*257* and IS*1182* close to these three genes may be the result of their insertion into the putative "ancestor" element (transposon, phage, or conjugative or mobilizable plasmid) responsible for their horizontal intergeneric transfer. Such a putative ancestor could be isolated by mating experiments with staphylococcal, enterococcal, and *Campylobacter* isolates carrying *aadE*, *sat4*, and *aphA-3* used as donors. The staphylococcal isolate having map IX, BM3235 (Table 1), which does not contain IS*1182* but carries all of the genes and ORFs found in the internal part of Tn*5405*, is a good candidate. The insertion sequences IS*1182* and IS*257* may also have been involved in the mobility of the three antibiotic resistance genes as a result of the in vivo construction of composite transposons such as Tn*5405* (5, 8). These genes may also spread via insertion sequence-mediated recombination events consistent with the presence of multiple copies of these two insertion sequences in the genome of all of the tested isolates carrying them.

ACKNOWLEDGMENTS

We thank Keith Dyke for constructive criticism and C. Tran for secretarial assistance.

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