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Diversity among the Gram-Positive Acetyltransferases Inactivating Streptogramin A and Structurally Related Compounds and Characterization of a New Staphylococcal Determinant, *vatB*

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A gene encoding an acetyltransferase inactivating streptogramin A (SgA) and structurally similar compounds was isolated from a staphylococcal plasmid and sequenced. This gene, designated *vatB*, potentially encodes a 212-amino-acid protein, VatB, of 23,320 Da with 47.4 and 58.4% amino acid identities with two other enzymes with the same activity, Vat and SatA, respectively, which are encoded by a staphylococcal plasmid and an enterococcal plasmid, respectively. The C-terminal parts of these three enzymes share significant homology with the C-terminal parts of 10 other acetyltransferases modifying various substrates. A pair of degenerate primers representing the conserved motifs shared by VatB, Vat, and SatA was designed to detect the three genes encoding these SgA acetyltransferases. Five of 12 clinical SgA^r *Staphylococcus aureus* isolates tested carried neither these genes nor the gene *vga*, which confers resistance to SgA by a different mechanism, suggesting that another gene(s) and possibly another mechanism of resistance to SgA in staphylococci remains to be characterized.

The antibiotics of the virginiamycin family produced by streptomycetes have one of two basic primary structures, A and B (12). Compounds of the A group, such as streptogramin A (SgA), pristinamycin IIA (PIIA), virginiamycin M, mikamycin A, or synergistin A, are polyunsaturated cyclic peptolides. Compounds of the B group, such as streptogramin B (SgB), pristinamycin IB, virginiamycin S, mikamycin B, or synergistin B, are cyclic hexapeptides. The group A and B compounds occur naturally in association. These associations (pristinamycin, virginiamycin, staphylomycin, mikamycin) have been used in Europe as oral antistaphylococcal agents. Their efficacy derives from the synergism of the group A and group B components. A semisynthetic injectable streptogramin (Synercid; RP 59500), which is made up of a mixture of group A and group B compounds (RP 54476 and RP 57669, respectively), is under study [J. Antimicrob. Chemother. **30**(Suppl. A), entire volume, 1992].

In France, resistance to the mixtures of group A and B compounds was first encountered among staphylococci in 1975 (17, 20, 25). To date, the prevalence of such strains remains low in most French hospitals (≤5%) (21, 23). Outbreaks caused by the dissemination of epidemic *Staphylococcus epidermidis* strains resistant to group A compounds have occasionally contributed to an increase in such strains in intensive care units (24, 39, 64). In staphylococci, resistance to mixtures of group A and B compounds (MIC of pristinamycin, ≥2 μg/ml) is always associated with resistance to group A compounds (MIC of SgA, ≥8 μg/ml) but not necessarily with resistance to group B compounds (21–24). Resistance to group A compounds is often, but not always, associated with in vitro resistance to the mixtures. In contrast, no significant increase in the in vitro resistance to the mixtures is observed for staphylococci resistant to SgB but susceptible to SgA (21). Two genes encoding resistance to group A compounds by distinct mechanisms, *vga* (1) and *vat* (3), and a gene encoding a lactonase that inactivates group B compounds, *vgb* (2, 22), have been detected on a *Staphylococcus aureus* plasmid, pIP680, conferring resistance to group A and B compounds and to their mixtures. The gene *vga*, which is also detected on *S. epidermidis* plasmids (39), encodes an ATP-binding protein that is probably involved in the active transport of group A compounds. The gene *vat* encodes an acetyltransferase inactivating SgA (and related compounds) and exhibits significant homology (58.5% amino acid identity) with the *Enterococcus faecium* acetyltransferase that inactivates group A compounds and that is encoded by the gene *satA* (52).

We report here the cloning and sequencing of a second staphylococcal gene, *vatB*, which encodes an acetyltransferase inactivating group A compounds. This enzyme is related to a family of acetyltransferases modifying various substrates. We also describe degenerate primers designed to detect by PCR three genes, *vat*, *vatB*, and *satA*, described to date that encode acetyltransferases inactivating group A compounds.

MATERIALS AND METHODS

Bacteria and plasmids. Table 1 lists the strains and plasmids used in the study.

Media. Brain heart infusion (BHI) broth and BHI agar (Difco Laboratories, Detroit, Mich.) were used for *S. aureus* growth and mating experiments. Susceptibility was tested by growing the bacteria on Mueller-Hinton agar (MHA; Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). Trypticase soy broth (TSB) or Trypticase soy agar (TSA) (Difco Laboratories, Detroit, Mich.) was used for the detection of SgA inactivation or acetylation. *Escherichia coli* was grown on Luria broth (LB) containing 10 g of tryptone per liter, 5 g of yeast extract per liter, and 10 g of sodium chloride per liter. Solid media contained 15 g of agar per liter. Bacteria were incubated at 37°C and, for liquid media, with agitation.

Susceptibilities to antimicrobial agents. Susceptibilities to antimicrobial agents were determined by a disk diffusion assay and with commercially available antibiotic disks (Diagnostics Pasteur). Additional disks prepared in our laboratory contained SgA (20 μg) or SgB (40 μg). The markers tested were those which

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TABLE 1. Strains and plasmids[a]

Strain or plasmid	Species	Relevant characteristics or source[b]	Resistance phenotype	Reference
Strains				
RN4220	*S. aureus*	Derivative of NTCC 8325		35
ISP1127	*S. aureus*	Recipient strain	Nv	48
TG1	*E. coli*	Derivative of K-12		27
DB11	*E. coli*	SgA susceptible mutant		12a
BM3093	*S. aureus*	Transductant obtained by using BM3041 as donor and BM225 as recipient	SgB SgA Pt Rf Fa Nv	22
BM3247	*S. aureus*	Clinical isolate	L SgA Pt Pc Mc Mn Tc As Cd Hg Km Gm Tm Nm Sm Sp Su	This study
BM3248	*S. aureus*	Clinical isolate	L SgA Pt Pc As Cd Hg Km Gm Tm	This study
BM3249	*S. aureus*	Clinical isolate	L SgA Pt Pc Mc Mn Tc As Cd Km Gm Tm Sm Su	This study
BM3250	*S. aureus*	Clinical isolate	L SgA Pt Pc Mc As Cd Hg Km Gm Tm Sm Su	This study
BM3251	*S. aureus*	Clinical isolate	L SgA Pt Pc Mc As Cd Hg Km Gm Tm	This study
BM3252	*S. aureus*	Clinical isolate	L SgA Pt Pc Mc Mn Tc Hg As Cd	This study
BM3253	*S. aureus*	Clinical isolate	L SgA Pt Pc Mc Mn Tc As Cd Hg Km Gm Tm Nm Sm Sp	This study
BM3385	*S. aureus*	Clinical isolate	L SgA Pt Pc Tp Mc As Cd Rf Su	This study
BM9023	*S. aureus*	Clinical isolate	M L SgB SgA Pt Pc Mn Tc Km Gm Tm Sm	This study
BM9260	*S. aureus*	Clinical isolate	M L SgB SgA Pt Pc Mn Tc Km Tm	This study
BM10155	*S. aureus*	Clinical isolate	M L SgB SgA Pt Pc Mc Tc As Cd Hg Km Tm Nm Sm	This study
BM10703	*S. aureus*	Clinical isolate	M L SgB SgA Pt Pc Mc Cd As Km Gm Tm Nm Sm Sp	This study
Plasmids				
pIP680 (11.4)[c]		BM3093	SgA SgB Pt	22
pIP1156 (≈60)		Transconjugant obtained by using BM3385 as donor and ISP1127 as recipient	Tp Pc L SgA Pt	This study
pIP1633 (≈50)		Transconjugant obtained by using BM3385 as donor and ISP1127 as recipient; deleted derivative of pIP1156	Pc L SgA Pt	This study
pUC18 (2.7)		Cloning vector	Ap	63
pOX300 (6.4)		Shuttle vector	Ap (*E. coli*) MLS (*S. aureus*)	19
pIP1689 (11.9)		pOX300 *Bam*HI Ω5.5-kb *Bgl*II fragment from pIP1633	Ap (*E. coli*) MLS, SgA, Pt (*S. aureus*)	This study
pIP1675 (8.2)		pUC18 *Bam*HI Ω5.5-kb *Bgl*II fragment from pIP1633	Ap	This study
pIP1677 (4.1)		pIP1675 Δ4.1-kb *Bgl*II-*Eco*RI fragment	Ap	This study
pIP1652 (3.3)		pUC18 *Sma*I Ω615-bp fragment of *vat* gene	Ap	This study
pIP1653 (3.0)		pUC18 *Sma*I Ω340-bp fragment of *vga* gene	Ap	This study
pIP1692 (3.3)		pUC18 *Sma*I Ω601-bp fragment of *vatB* gene	Ap	This study

[a] Abbreviations (resistance): Ap, ampicillin; As, sodium arsenate; Cd, cadmium acetate; Fa, fusidic acid; Gm, gentamicin; Hg, mercuric nitrate; Km, kanamycin; L, lincosamides; Mc, methicillin; MLS, macrolides-lincosamides-streptogramin B; Mn, minocycline; Nm, neomycin; Nv, novobiocin; Pc, penicillinase production; Pt, pristinamycin (MICs, ≥2 μg/ml); Rf, rifampin; SgA, streptogramin A (MICs, ≥8 μg/ml); SgB, streptogramin B; Sm, streptomycin; Sp, spectinomycin; Su, sulfonamide; Tc, tetracycline; Tm, tobramycin; Tp, trimethoprim.

[b] The clinical isolates included in the study were considered unrelated either because their *Sma*I profiles differed by more than five fragments or because the percentages of similarity between the hybridization patterns of the *Eco*RI-digested cellular DNA with an IS*256* probe (39) were ≤35.

[c] Values in parentheses are sizes (in kilobases).

enabled us to detect the drug resistance phenotypes described to date among staphylococci.

The MICs of the antibiotics were determined by serial twofold dilutions of antibiotics in MHA (26).

Detection of PIIA inactivation or acetylation. Inactivation of PIIA was investigated by the Gots test, with *Micrococcus luteus* ATCC 9341 used as the indicator organism, by using an SgA (Rhône-Poulenc Rorer, Vitry S/Seine, France) concentration of 0.2 μg/ml in the media containing TSA (3). To investigate the mechanism of inactivation, the strain was grown at 37°C for 18 h in 1 liter of TSB containing 20 μg of PIIA. The cells were harvested by centrifugation, and the supernatant was extracted with chloroform (three times with 300 ml each time). After evaporation of the solvent, the residue was dissolved in 1 ml of chloroform. Thick-layer chromatography was performed on Kiesel gel 60F plates (Merck); the eluent was a mixture of chloroform and methanol (87:13). A reference consisting of 100 μg of PIIA dissolved in methanol was deposited on the same plate. The intact PIIA and the modified PIIA were visualized at 254 nm. The gel containing these compounds was collected and treated with 5 ml of chloroform. Purified extracts recovered after chloroform evaporation were analyzed by nuclear magnetic resonance at 25°C with a Unity Varian spectrometer operating at 500 MHz for the proton. The samples were dissolved in deuterated chloroform, and chemical shifts were calibrated with the solvent nuclear magnetic resonance line (δ = 7.24 ppm) (3).

Mating procedure. A 0.3-ml portion of a mixture containing 10^7 CFU of donors and 10-fold more recipients, both obtained from late-logarithmic-phase broth cultures, was spread onto nitrocellulose filters (type HAEP; pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). After 18 h of incubation at 37°C, the cells from each filter were suspended in 1 ml of BHI broth, and the suspension was spread onto BHI agar containing the appropriate selective drug. Controls consisting of donor or recipient cells alone were treated similarly. SgA (20 μg/ml) was used to select the donor markers, and novobiocin (25 μg/ml) was used to counterselect bacterial donors.

DNA isolation and analysis. Total cellular DNA was isolated from staphylococcal strains and was purified as described previously (13). The technique used to extract plasmids from the staphylococci (15) was adapted from that of Portnoy et al. (50). Plasmid DNA was purified in a cesium chloride-ethidium bromide density gradient. Plasmid DNA was isolated from *E. coli* by a rapid alkaline

TABLE 2. Relevant characteristics of the oligonucleotides used in PCR experiments

Oligonucleotide	Sequence	Location in the published gene sequence	Size (nt) of amplified DNA fragment
A	5′-CAATGACCATGGACCTGATC-3′	nt 12–nt 31 within *vat* (3)	615
B	5′-AGCATTTCGATATCTCC-3′	nt 610–nt 626 within *vat*	
C	5′-CCTATAGAAGGAAACAAATCAG-3′	nt 28–nt 49 within *satA* (52)	595
D	5′-CCATATGACTTCTCTAATGATGC-3′	nt 600–nt 623 within *satA*	
E	5′-GTIGGIGAATACTCITACTACGAC-3′ G TA T T T	Cf. Fig. 1 (this study)	285
F	5′-ACATCCCTICCIATCCAIACATC-3′ G TT G		
G	5′-CCTGATCCAAATAGCATATATCC-3′	nt 13–nt 35 within *vatB* (this study)	601
H	5′-CTAAATCAGAGCTACAAAGTG-3′	nt 591–nt 613 within *vatB*	
I	5′-ATTATGAATGGAGCAAACCATAGAATG-3′ T T C G	Cf. Fig. 5 (this study)	144 from within *vat* and *satA*
J	5′-ACCAATCCACACATCATTTCC-3′ T T T A		147 from within *vatB*
K	5′-CCAGAACTGCTATTAGCAGATGAACC-3′	nt 290–nt 315 within *vga* (1)	474
L	5′-GGAAGTTCGTTTCTCTTTTCGACGC-3′	nt 740–nt 764 within *vga*	

extraction procedure (6). Large-scale plasmid DNA was purified by cesium chloride density gradient centrifugation of cleared lysates. DNA fragments extracted from agarose gels were purified with the GeneClean II kit (Bio 101, La Jolla, Calif.).

For PCR experiments, the cellular DNA was extracted and purified from a single colony by the procedure proposed by the manufacturer of the InstaGene DNA purification matrix (Bio-Rad, Hercules, Calif.).

Restriction endonucleases were obtained from Amersham International (Amersham, United Kingdom) or from Pharmacia (Uppsala, Sweden) and were used according to the manufacturers' instructions. Digested DNA was subjected to electrophoresis in agarose (0.7% [wt/vol]) or in Nusieve agarose (4% [wt/vol]) (FMC Bioproduct, Rockland, Maine) in Tris-borate buffer as described by Sambrook et al. (54). The Raoul I ladder (Appligène, Strasbourg, France) and the 1-kb DNA ladder (Gibco BRL, Gaithersburg, Md.) were used as size markers.

PCR. DNA was amplified by PCR in a Crocodile II thermal cycler (Appligène) with approximately 10 ng of cellular DNA or 1 ng of plasmid DNA. The reaction mixture contained 0.6 μM (each) oligonucleotide serving as primer, 200 μM (each) deoxynucleotide triphosphate, 2.5 U of *Taq* DNA polymerase (Amersham International), and 1× buffer (Amersham International). The final reaction volume was adjusted to 100 μl with H_2O, and the sample was then covered with 50 μl of heavy white mineral oil (Sigma Chemical Co., St. Louis, Mo.).

PCR experiments were carried out at high or low stringency, depending on the primers used. At high stringency, PCR was performed with a precycle of 3 min at 95°C and 2 min at 60°C and 30 cycles of 20 s at 72°C, 20 s at 95°C, and 20 s at 60°C; these were followed by a cycle of 1 min at 72°C. At low stringency, PCR was performed with a precycle of 5 min at 95°C and 35 cycles of 2 min at 40°C, 1 min and 30 s at 72°C, and 30 s at 95°C; these were followed by a cycle of 4 min at 40°C and 12 min at 72°C. The oligonucleotides used in the present study are indicated in Table 2.

Blotting and hybridization. Hybond-N^+ membranes (Amersham International) were used for blotting. DNA was transferred from agarose gels to the membranes by the capillary blotting method of Southern (58). Prehybridization and hybridization were performed under stringent conditions as described previously (10). Purified linear DNA fragments extracted from agarose gel or plasmids, which were used as probes, were labelled with [α-^{32}P]dCTP (110 TBq $mmol^{-1}$) by the random priming technique with the Megaprime DNA labelling system (Amersham International).

DNA cloning and transformation. Standard methods were used for DNA cloning (54). Transformation of *E. coli* TG1 was accomplished by the method of Hanahan (28) with selection on LB agar containing ampicillin (100 μg/ml). Clones were screened for plasmids by the method of Birnboim and Doly (6).

S. aureus RN4220 was transformed by electroporation. BHI broth (200 ml) was inoculated with 1 ml of an overnight culture of *S. aureus* RN4220. Cells were grown at 37°C to an optical density at 600 nm of 0.4 and were harvested by centrifugation. The pellet was washed twice with sucrose (0.5 M)–HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; 1 mM) buffer and was resuspended in 2.5 ml of sucrose-HEPES buffer containing 10% glycerol. Aliquots (100 μl) of this suspension were frozen and stored at −80°C. For electroporation, 100 μl of cells was thawed on ice, and 10 μl of a solution containing 100 ng of recombinant plasmid was added. The mixture was transferred to an electroporation cuvette (0.2-cm interelectrode gaps), and a single electric pulse (Bio-Rad Gene Pulser apparatus) was applied (2.5-kV peak voltage, 25-ΩF capacitance, and 100-Ω resistance). One milliliter of BHI broth was added. After 1 h of incubation at 37°C, 200 μl of the suspension was spread onto BHI agar supplemented with the antibiotic used to select the transformants.

DNA sequencing. DNA was sequenced by the dideoxynucleotide chain-termination method (55). Double-stranded plasmid DNA was labelled with [α-^{35}S]dATP (Amersham International), and the T7 polymerase sequencing kit (Pharmacia LKB) was used according to the supplier's instructions. Two types of primers were used for sequencing: the M13 reverse sequencing primer (Pharmacia) and sequence-specific synthetic oligoprimers.

Sequence analysis. The amino acid sequence was analyzed with the GCG package. The amino acid sequence of the *vatB* gene was compared with those derived from the nucleotide (nt) sequences introduced in the GenBank-EMBL Data Library DNA databases by using the program TFastA. The amino acid sequences were aligned according to the algorithm delivered by the Clustal V package (30).

Nucleotide sequence accession number. The sequence data presented here were submitted to GenBank-EMBL Data Library and have the accession number L38809.

RESULTS

Cloning of *vatB* gene from plasmid pIP1633, a deleted derivative of plasmid pIP1156. Wild-type *S. aureus* BM3385 was crossed on a membrane filter with the *S. aureus* recipient strain ISP1127 (48). Two types of transconjugants, distinguishable by their drug resistance phenotypes, were selected on solid medium containing 20 μg of SgA per ml. Both types of transconjugants were resistant to group A compounds, the mixtures of group A and B compounds, lincomycin, and penicillins by penicillinase production. One of the two types of transconjugants was also resistant to trimethoprim. These trimethoprim-resistant strains harbored a plasmid, pIP1156, having the same size (≈60 kb) and *Eco*RI restriction profile as those of the plasmid carried by the donor strain. This plasmid was shown to inactivate PIIA by the Gots test, and nuclear magnetic resonance analysis revealed the presence of an *O*-acetyl group at position C-14 (3). A deleted derivative of pIP1156, pIP1633 (≈50 kb), was detected in one of the trimethoprim-susceptible transconjugants. Plasmids pIP1156 and pIP1633 both conferred the same level of resistance to SgA (MIC, 64 μg/ml).

Plasmids pIP1156 and pIP1633 were probed under stringent conditions with [α-^{32}P]dCTP-labelled pIP1652 containing a

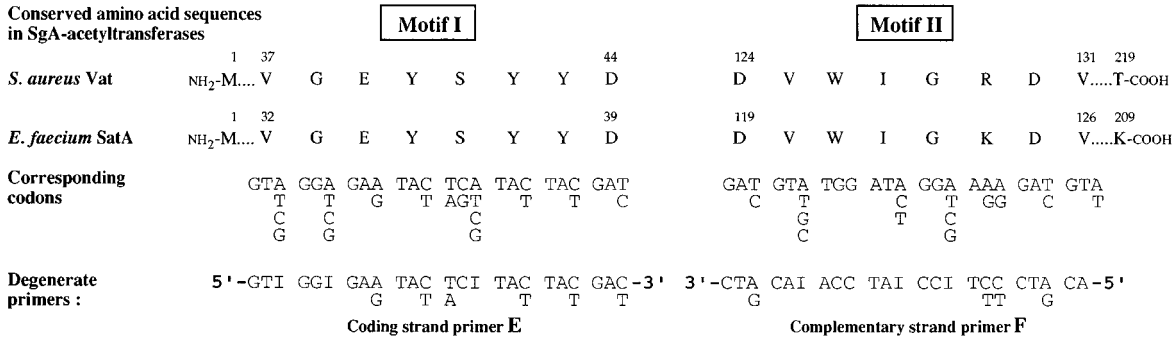

FIG. 1. Description of degenerate primers E and F used to amplify by PCR, at low stringency, fragments of *vat*-related genes. The relative positions of the amino acids in the two selected conserved motifs, motifs I and II, are those reported in the published sequences of *vat* (3) and *satA* (52). I, deoxyinosine.

615-bp DNA fragment of the *vat* gene (3). Neither of the plasmids hybridized with pIP1652; thus, it is unlikely that they carry the *vat* gene (data not shown). This observation prompted us to reinvestigate a result that we had reported previously (3), i.e., that the *vat* gene was detected in pIP1156 by a PCR experiment done at high stringency with oligonucleotides A and B used as primers. When the latter experiment was repeated with a new preparation of pIP1156, no amplification of any DNA fragment was observed, suggesting that the first DNA preparation had been contaminated with one of the several plasmids containing *vat* which were simultaneously being prepared in our laboratory. Nor was any amplification observed when the new preparation of pIP1156 was used as a template in a PCR experiment carried out at high stringency (annealing temperature, 60°C) with primers C and D, which have been used elsewhere to amplify a 595-bp DNA fragment from within the *E. faecium satA* gene (52).

A pair of degenerate oligonucleotides, oligonucleotides E and F, was designed to anneal, at low stringency (40°C), to all of the putative sequences that encode the conserved motifs I and II, each containing eight amino acids of the acetyltransferases encoded by *vat* and *satA*. These two motifs and their positions in the published sequences of each enzyme are indicated in Fig. 1, as are all of the possible codons which may encode the amino acid sequence of motifs I and II. When an amino acid may be encoded by two alternate codons only, the nucleotides, which differ between such codons, were provided in equimolar amounts during the synthesis of oligonucleotide E or F. Deoxyinosine, which can potentially pair with any DNA base, was used when more than two putative codons could encode a given amino acid. The designed oligonucleotides, oligonucleotides E (coding-strand primer) and F (complementary-strand primer), which had 4,096- and 1,024-fold degeneracies, respectively, were expected to be able to amplify a 285-bp DNA fragment from within the *vat* and *satA* genes.

Plasmid pIP680 containing *vat* and plasmid pIP1633 containing *vatB* were primed with oligonucleotides E and F in PCR experiments carried out at low stringency (40°C). A 285-bp DNA fragment was amplified from within each plasmid. These two fragments were extracted from an agarose gel, purified, labelled with [α-^{32}P]dCTP, and used as probes for high-stringency hybridization experiments. The fragment amplified from within pIP1633 hybridized with pIP1633 (Fig. 2A) but not with pIP680 (data not shown), whereas the fragment amplified from pIP680 hybridized with pIP680 but not with pIP1633 (data not shown). These results suggested that plasmid pIP1633 carried a *vat*-related gene encoding an acetyltransferase which, although containing at least part of the conserved motifs I and II, had sufficiently diverged to prevent annealing of the two genes under high-stringency conditions.

As shown in Fig. 2B, the nucleotide sequences hybridizing with the 285-bp DNA fragment of pIP1633 were detected on a single restriction fragment of pIP1633, i.e., a 9-kb *Hin*dIII fragment and a 5.5-kb *Bgl*II fragment, whereas fragments of 1.4 and 0.4 kb hybridizing with this probe were detected when pIP1633 was cleaved with *Hin*dIII plus *Eco*RI. These results suggested that the suspected *vat*-related gene of pIP1633 contains, by contrast with the *vat* gene (3), at least an *Eco*RI restriction site.

The 5.5-kb *Bgl*II fragment of pIP1633, which was thought to carry a *vat*-related gene, was selected for cloning. For this purpose, *Bgl*II-cleaved pIP1633 was ligated with the *Bam*HI-cleaved shuttle vector pOX300 (19), a hybrid of pUC18 (62) and pE194ts (32), which replicates in *E. coli*, in which it confers resistance to ampicillin, and in *S. aureus*, in which it confers

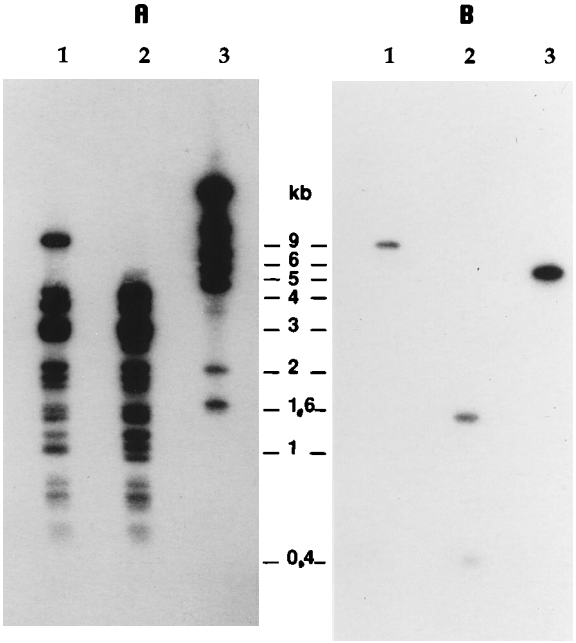

FIG. 2. Hybridization patterns of plasmid pIP1633 cleaved with *Hin*dIII (lanes 1), *Hin*dIII plus *Eco*RI (lanes 2), or *Bgl*II (lanes 3) and probed with [α-^{32}P]dCTP-labelled pIP1633 (A) or the 285-bp DNA fragment of the *vat*-related gene carried by pIP1633 (B).

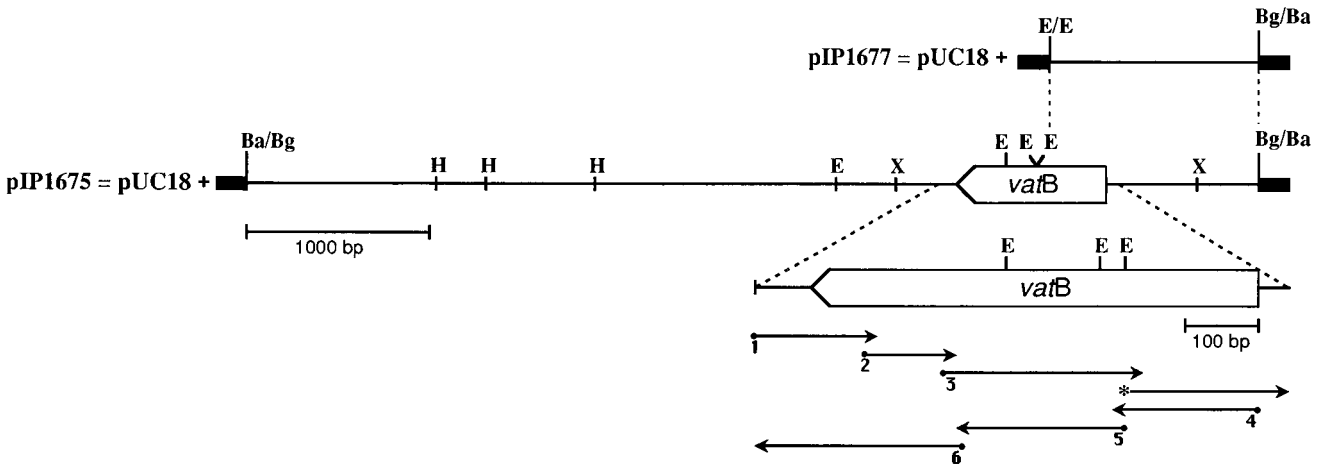

FIG. 3. Restriction map of the 5.5-kb *Bgl*II fragment from pIP1633 conferring resistance to SgA and related compounds and strategy for sequencing the *vatB* gene. Arrows indicate the direction and extent of each dideoxy-sequencing reaction. The asterisk designates the reverse sequencing primer (Pharmacia International), and the dots numbered 1 to 6 designate the sequence-specific oligonucleotide primers, as follows: 1, 5′-GAATTGAAAAGCCTAAATA-3′; 2, 5′-CCGATGTTAACGGA GCTG-3′; 3, 5′-ATGGAAATGGTTGGGA-3′; 4, 5′-CGTGTGTAAGTTCTTAG-3′; 5, 5′-CTCGGATTTCCACC-3′; 6, 5′-GAATTCATTCTATGG-3′. Restriction enzyme abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I.

constitutive resistance to macrolides, lincosamides, and SgB. The ligation mixture was used to transform *E. coli* TG1. The Ap^r transformants were screened by colony hybridization under stringent conditions with a probe consisting of the labelled 285-bp DNA fragment amplified from pIP1633 with oligonucleotides E and F. The recombinant plasmid, pIP1689, which was isolated from an Ap^r transformant and which hybridized with the probe, consisted of pOX300 plus a *Bgl*II insert of 5.5 kb. This recombinant plasmid was introduced by transformation into *E. coli* DB11, which exhibits a lower level of resistance to group A compounds (SgA MIC, 4 μg/ml), and by electroporation into *S. aureus* RN4220 (SgA MIC, 2 μg/ml). The *E. coli* transformants were selected on LB agar containing 100 μg of ampicillin per ml, and the *S. aureus* transformants were selected on BHI agar containing 10 μg of erythromycin per ml. Plasmid pIP1689 significantly increased the resistance of *S. aureus* to SgA (MIC, 64 μg/ml), suggesting that the 5.5-kb *Bgl*II insert of pIP1689 carried the *vat*-related gene of pIP1633. The level of resistance to SgA conferred by pIP1689 was similar to that conferred by pIP1633 (64 μg/ml). By contrast, pIP1689 did not significantly modify the level of resistance of *E. coli* DB11 to SgA.

Sequencing of the *vat*-related gene carried by pIP1633. Two recombinant plasmids, pIP1675 and pIP1677, were constructed to serve as templates for sequencing the *vat*-related gene carried by the 5.5-kb *Bgl*II fragment cloned from pIP1633. Plasmid pIP1675 (Fig. 3) resulted from the ligation of the 5.5-kb *Bgl*II fragment from pIP1633 into pUC18 cleaved with *Bam*HI. Cleavage of plasmid pIP1675 with *Eco*RI and then ligation yielded plasmid pIP1677 (Fig. 3) containing a 1.2-kb *Eco*RI-*Bgl*II insert, which was used to begin sequencing with the M13 reverse sequence primer (Pharmacia), close to the *Eco*RI restriction site thought to be carried by the *vat*-related gene of pIP1633.

The strategy of sequencing on both strands is outlined in Fig. 3, and the sequence of the 800-bp fragment from within the 5.5-kb *Bgl*II fragment of pIP1633 is reported in Fig. 4. The open reading frames (ORFs) and the putative genes were searched in the six reading frames taking into account three possible start codons (ATG, GTG, and TTG). Only one ORF (660 nt), located between nt 43 and nt 702, that contains a putative gene was detected. This ORF contained a single start codon (ATG) at nt 67 preceded 6 nt upstream by a 7-nt putative ribosome-binding site (RBS) (42–44) (Fig. 4). The free energy of association of the most stable structure between the putative RBS and the 3′ terminus of the 16S rRNA calculated according to Tinoco et al. (60) was −78.8 kJ/mol. The sequence located between the start ATG codon at nt 67 and nt 702 preceding the stop codon TAG may encode a 212-amino-acid protein of 23,320 Da. The G+C content of this putative gene, named *vatB*, is 31.4%. This value is slightly lower than that of the gene *vat* (35.3%) (3) but similar to that of the gene *satA* (30.8%) (52) and to the overall value for the staphylococcal genome (32 to 36%) (34).

We did not find any structure suggesting a translational regulation involving a leader peptide (40, 41) upstream of the ATG codon of *vatB*, any putative promoter consensus sequences rich in A+T because the A+T content of this gene is high, or any structure which might function as a Rho-independent transcriptional terminator (49). The *vatB* gene exhibited only 53.3% nt identity with the gene *vat* (3) and 52.6% nt identity with the gene *satA* (52).

Analysis of the VatB amino acid sequence. The amino acid composition of the predicted translation product of the *vatB* gene, VatB, indicates that it is acidic (40 Tyr, Cys, Glu, and Asp versus 26 Arg, Lys, and His) and has a calculated pI of 5.18. The hydropathy plot (36) of the VatB amino acid sequence revealed a hydrophilic protein without similarity to known signal sequences of the secreted proteins (63).

The amino acid sequence of VatB was compared with those derived from the nt sequences introduced in the GenBank-EMBL Data Library by using the program TFastA. The overall peptide sequence of VatB exhibited significant homology with the overall peptide sequences of the proteins encoded by *S. aureus vat* (3) (50.4% amino acid identity; Fig. 5), *E. faecium satA* (52) (47.4% amino acid identity; Fig. 5), *Agrobacterium tumefaciens cat* (59) (33.9% amino acid identity), *E. coli cat* (47) (33.3% amino acid identity), and the peptide sequence deduced from a partial ORF (Bsorf) located upstream of the *Bacillus sphaericus* chromosomal *ermG* gene (43) (50.5% amino acid identity). In addition, the carboxyl end of VatB (amino acids 118 to 170) exhibited amino acid identities with

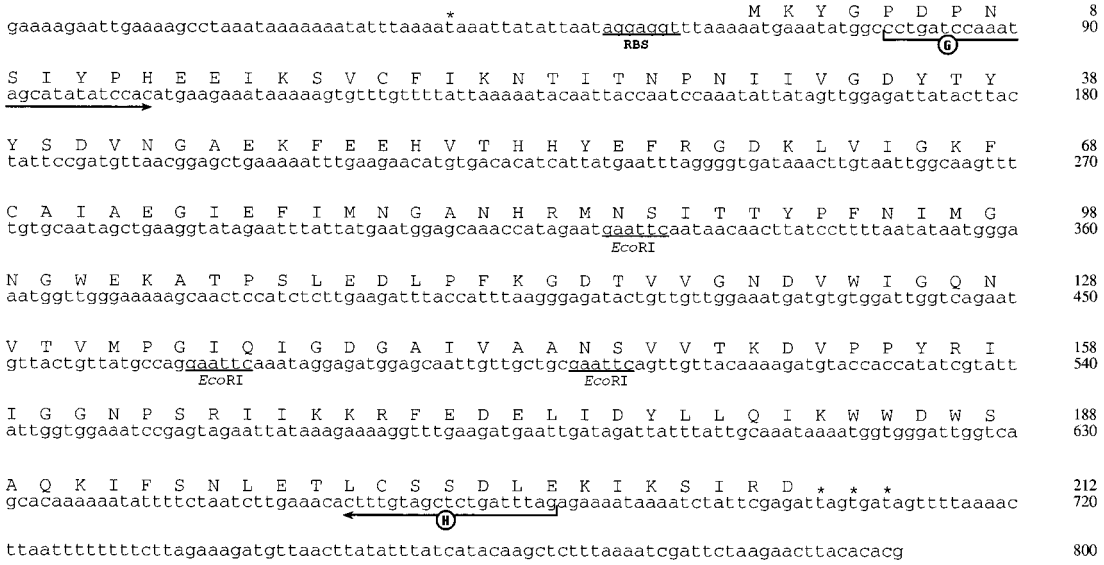

FIG. 4. Nucleotide sequence and deduced amino acid sequence of 800 nt from pIP1633, which contains the gene *vatB* conferring to *S. aureus* resistance to SgA and related compounds. The putative RBS is underlined. The stop codons are indicated by asterisks. Oligonucleotides G and H, which were used to amplify at high stringency a DNA fragment from within *vatB*, are underlined.

the C-terminal regions of 10 other acetyltransferases modifying various substrates ranging from 33.7 to 43.9% (Fig. 6). The consensus sequence consisted of 19 amino acids present in at least 10 of the 13 aligned proteins (Fig. 6). No significant amino acid homology was detected with the remaining acetyltransferases described to date, including the staphylococcal chloramphenicol acetyltransferases (57).

Analysis of 11 clinical SgA^r *S. aureus* isolates which do not carry the *vat* or *vga* gene. Similar to BM3385, neither of the two *S. aureus* SgA^r genes described previously, *vga* (1) or *vat* (3), was detected in the cellular DNA of the 11 remaining clinical *S. aureus* SgA^r isolates studied (Table 1). The presence of both genes was investigated by hybridization experiments with probes containing DNA fragments from within *vga* or *vat*, pIP1653 and pIP1652, respectively, and by PCR experiments at high stringency with the pairs of primers K-L and A-B (data not shown). The recombinant plasmid pIP1692 containing a fragment of *vatB* was labelled with [α-^{32}P]dCTP and was also used to probe the cellular DNA of the 12 clinical SgA^r isolates. BM3385, as well as six of the isolates (BM3247, BM3248, BM3251, BM3253, BM9023, and BM9260), hybridized, whereas no hybridizing nucleotide sequences were detected in the DNAs of the five other strains (BM3249, BM3250, BM3252, BM10155, and BM10703) (data not shown). None of the five strains which do not contain the genes investigated exhibited inactivation of PIIA detectable by the Gots test. Moreover, primers G and H, designed to amplify a 601-bp DNA fragment from within *vatB*, were used in PCR experiments at high stringency. A fragment of the expected size was amplified from the cellular DNAs of the former strains, whereas no DNA product was amplified from those of the last five strains (data not shown). The degenerate primers E and F encoding the con-

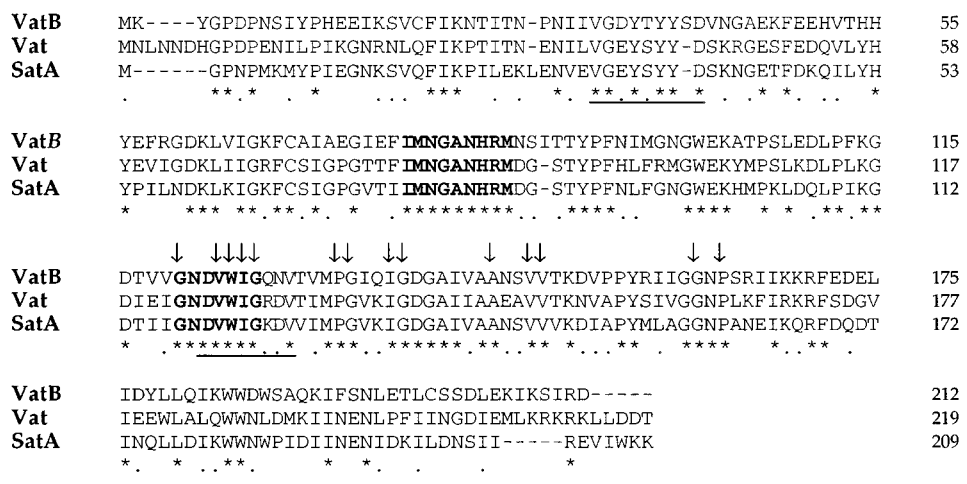

FIG. 5. Amino acid similarities among VatB, Vat (3), and SatA (52). The amino acids given in boldface type are the conserved motifs III and IV selected for the design of degenerate primers I and J (see Fig. 7) with which we amplified 144- to 147-nt fragments from each of the three genes. Identical residues are indicated by asterisks, and conserved changes are indicated by single dots. The 14 amino acids indicated by arrows are those in the consensus sequence found in a family of related acetyltransferases (see Fig. 6). Motifs I and II, which were used to detect VatB in the present study, are underlined.

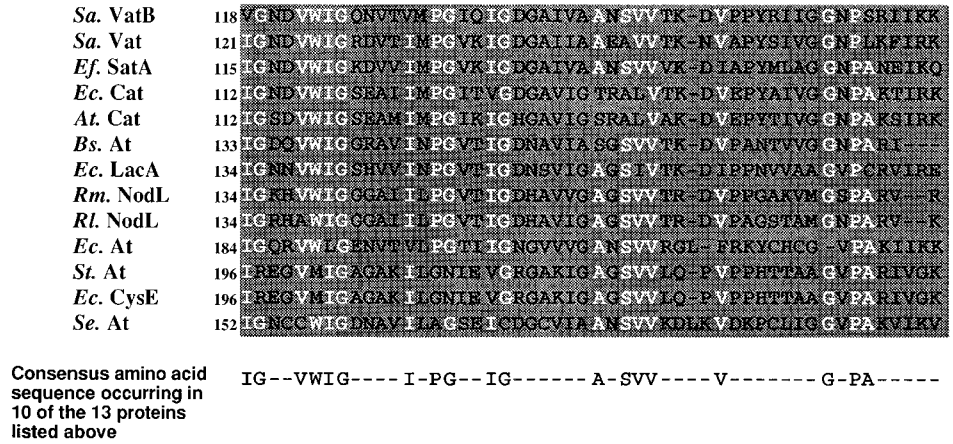

FIG. 6. Alignment of the carboxyl ends of the amino acid sequences of 13 acetyltransferases exhibiting significant homologies: *Sa*. VatB, *S. aureus* acetyltransferase inactivating SgA and related compounds (the present study); *Sa*. Vat, *S. aureus* acetyltransferase inactivating SgA and related compounds (3); *Ef*. SatA, *E. faecium* acetyltransferase inactivating SgA and related compounds (52); *Ec*. Cat, *E. coli* chloramphenicol acetyltransferase (47); *At*. Cat, *Agrobacterium tumefaciens* chloramphenicol acetyltransferase (59); *Bs*. At, *Bacillus subtilis* putative acetyltransferase (51); *Ec*. LacA, *E. coli* thiogalactoside acetyltransferase (29); *Rm*. NodL, *Rhizobium meliloti* putative acetyltransferase (5); *Rl*. NodL, *Rhizobium leguminosarum* putative acetyltransferase (16); *Ec*. At, *E. coli* putative acetyltransferase (66); *St*. At, *Salmonella typhimurium* serine acetyltransferase (53); *Ec*. CysE, *E. coli* serine acetyltransferase (14); *Se*. At, *Salmonella enterica* putative acetyltransferase (8).

served motifs I and II detected in Vat and SatA at low stringency did not detectably amplify the cellular DNAs of any of the last five strains. These results prompted us to design the degenerate oligonucleotides I and J that prime the DNA amplification of sequences within the *vat*, *vatB*, or *sat* gene.

As shown in Fig. 5, only part of the amino acid sequence in motifs I and II reported in Fig. 1 was detected in the peptide sequence of VatB. Thus, two other highly conserved motifs, motifs III and IV, were selected by alignment of the amino acid sequences of the acetyltransferases Vat, VatB, and SatA (Fig. 5). The degenerate primers I and J, designed by taking into account the nt sequences of the three genes encoding motifs III and IV, are described in Fig. 7. The 144-nt fragment amplified from *vat* carries a *Bgl*II site generating 97- and 47-bp fragments, whereas the 147-nt fragment of *vatB* carries an *Eco*RI site which delimits 117- and 30-bp fragments. Neither of these two restriction sites are present in the 144-nt fragment in the *satA* gene. Primers I and J were used to investigate the three genes encoding SgA acetyltransferases in the 12 clinical SgA^r staphylococcal isolates and in the transductant carrying *vat*, BM3093. PCR experiments carried out at high and low stringencies yielded amplification of a DNA fragment of the expected size (144 to 147 nt) from BM3093 (Fig. 8, lane 14), BM3385 (Fig. 8, lane 13), and the six clinical isolates carrying *vatB* (Fig. 8, lanes 1 to 6), whereas no amplified products were observed with the five remaining isolates (Fig. 8, lanes 8 to 12). The 147-nt fragments amplified from the seven clinical isolates, including BM3385, carried an *Eco*RI site at the expected location. Moreover, recombinant plasmid pIP1692 hybridized with the seven 147-nt amplified fragments but not with the amplified fragment of the *vat* gene carried by transductant BM3093 (Fig. 8B). None of the 147-nt fragments amplified from within *vatB* hybridized with pIP1652, which contained an insert from within *vat* (Fig. 8C).

DISCUSSION

The staphylococcal *vatB* gene, which has been described in this report and which has been shown to encode resistance to SgA and similar compounds, appears to be related by no more than 56.4% nt identity to the two other genes characterized previously, *vat* and *satA*, which encode proteins that have the same enzymatic activity and which were isolated from *S. aureus* (3) and *E. faecium* (52), respectively. The deduced amino acid sequences of these three genes are related by 47.4 to 58.5% identity. The fact that plasmid or chromosomal antibiotic re-

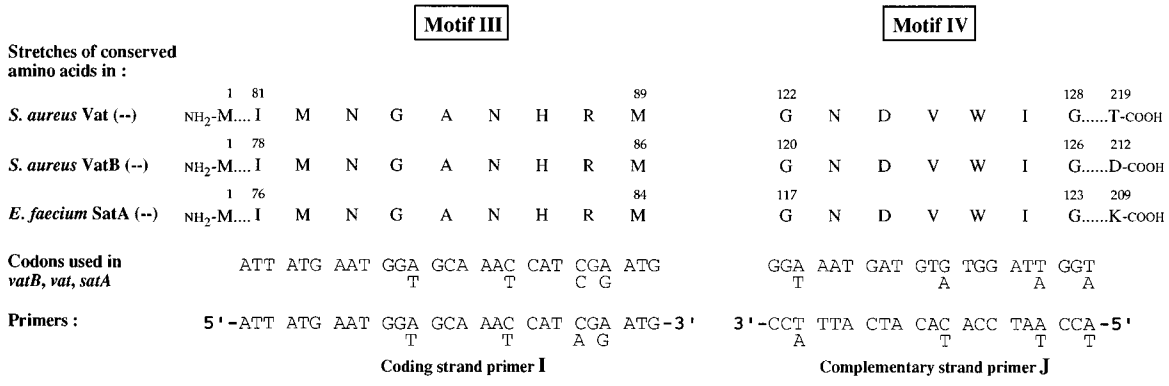

FIG. 7. Description of the degenerate primers I and J designed to amplify 144- to 147-nt DNA fragments from within the genes *vat* (3), *vatB* (the present study), and *satA* (52) encoding acetyltransferases inactivating SgA and related compounds.

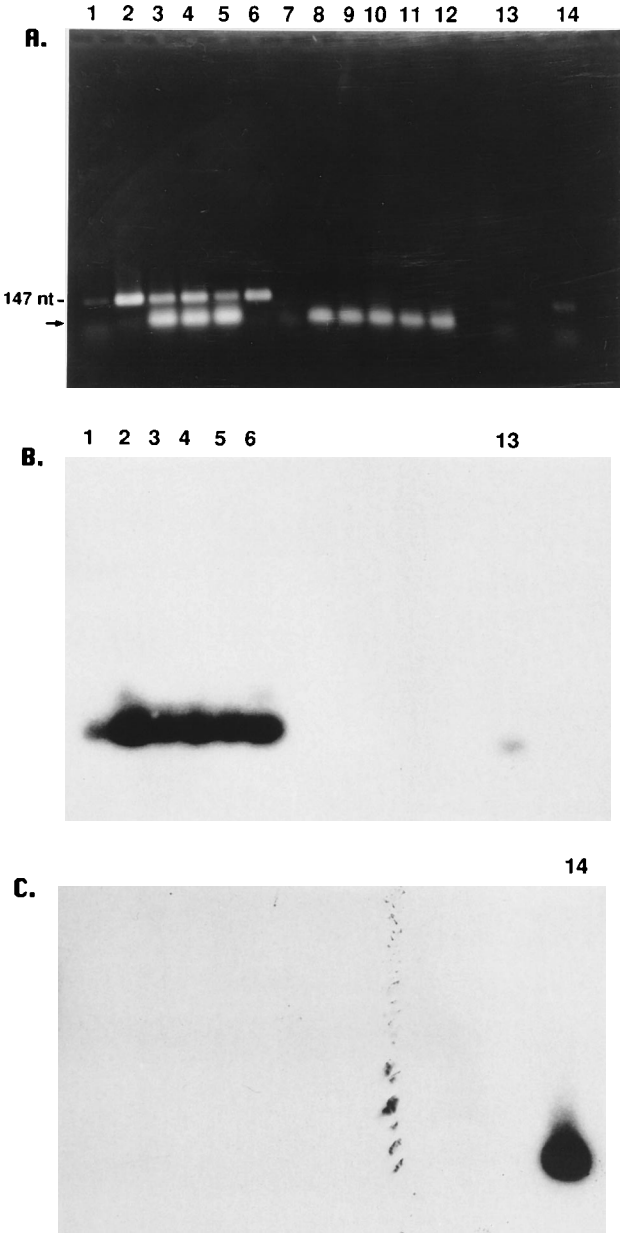

FIG. 8. PCR products obtained with oligonucleotides I and J (A) and hybridization patterns with the following probes: pIP1692 containing a 601-bp insert from within *vatB* (B) and pIP1652 containing a 614-bp insert from within *vat* (C). Lanes: 1, BM3247; 2, BM3248; 3, BM3251; 4, BM3253; 5, BM9023; 6, BM9260; 7, no DNA; 8, BM3249; 9, BM3250; 10, BM3252; 11, BM10155; 12, BM10703; 13, BM3093; 14, BM3385. The bands indicated by an arrow correspond to the primers.

sistance genes carried by enterococci can be transferred by conjugation in vitro into *S. aureus* recipients (9, 11, 33, 46), as well as the fact that closely related genes or transposons are present in enterococci and staphylococci (4, 7, 18, 31, 37, 45, 56, 61, 65), suggests that genetic exchange may occur between these two genera in vivo. The three SgA^r genes described to date may be distant derivatives of a parental gene inherited long ago by a staphylococcal or an enterococcal isolate. Alternatively, the relatively high degree of divergence between these genes may reflect an independent, horizontal inheritance from various sources.

Each of the three genes encoding acetyltransferases inactivating SgA and described to date, *vat* (3), *vatB* (this report), and *satA* (52), was isolated from a plasmid. The *vat* gene, which was cloned from plasmid pIP680, was shown to be contiguous to the *vgb* gene encoding a lactonase inactivating SgB and related compounds (2). Moreover, these two genes are flanked by two inverted copies of the insertion sequence IS*257*, which delimit a 5.3-kb DNA fragment also detected in two structurally different plasmids harbored by independent *S. aureus* and *Staphylococcus simulans* isolates (21, 38). The plasmids carrying *vat*, *vgb*, and *vga* are not transferable by conjugation, whereas plasmid pIP1156, which contains the *vatB* gene, is. None of the seven clinical staphylococcal isolates containing *vatB* included in the present study carries the *vgb* gene; five of these isolates have an uncommon drug resistance phenotype: they are resistant to SgA, pristinamycin, and lincomycin but are susceptible to macrolides and SgB (Table 1). In the absence of selection on media containing SgA, some of these strains exhibited a low level of resistance to SgA (MICs, 8 to 16 μg/ml) and appeared to be susceptible in vitro to pristinamycin (MICs, ≤1 μg/ml). The structural instability of the plasmids carrying *vatB* may contribute to the loss of *vatB* in part of the bacterial population. Such an instability has also been observed among the staphylococcal plasmids carrying *vat-vgb-vga* or *vga* alone (21, 38). Although strains harboring such plasmids appear to be susceptible in vitro to the synergistic associations of group A and B compounds, these drugs should not be used to treat infections caused by staphylococcal isolates carrying such plasmids. The presence of such plasmids may be detected by investigating resistance to SgA rather than resistance to the association by using disks containing 20 μg of this antibiotic, selection on solid medium containing 10 μg of SgA per ml, or detection of genes for SgA^r. The detection of two conserved motifs, motifs III and IV, in the amino acid sequence of Vat (3), VatB (the present study), and SatA (52) enabled us to design a degenerate pair of primers, primers I and J, to detect the genes encoding these three proteins. None of the SgA^r genes investigated, including *vga*, was detected in 5 of the 11 SgA^r staphylococcal isolates tested. These five strains may be mutants or may harbor genes for SgA^r that have not yet been characterized.

The C termini of the SgA acetyltransferases encoded by *vat*, *vatB*, and *satA* exhibit significant homology with the C-terminal parts of 10 other acetyltransferases modifying various substrates. The consensus amino acid sequences detected in the C-terminal parts of at least 10 of the 13 acetyltransferases aligned in Fig. 6 are probably involved in the interaction between these enzymes and acetyl coenzyme A, which is an acetyl donor involved in the modification of the chloramphenicol by the chloramphenicol acetyltransferases (57). The number of amino acids in the consensus sequence reported in Fig. 6 (19 amino acids) is smaller than that reported by Downie (16) and Parent and Roy (47) (29 amino acids) because of the alignment of a larger number of acetyltransferases in our study. This family of acetyltransferases includes some chloramphenicol acetyltransferase proteins which do not exhibit any significant homology with the chloramphenicol acetyltransferases detected in *S. aureus*, *Bacillus pumilus*, *Clostridium difficile*, or *Streptomyces acrimycini* (57). Consequently, X-ray diffraction studies of crystals of one of the acetyltransferases described in Fig. 6 will be necessary if the mode of interaction of the enzyme with acetyl coenzyme A and the substrate is to be elucidated.

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