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Genetic Analysis of Gentamicin Resistance in Methicillin- and Gentamicin-Resistant Strains of *Staphylococcus aureus* Isolated in Dublin Hospitals

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Methicillin- and gentamicin-resistant strains of *Staphylococcus aureus* isolated in Dublin hospitals have been classified into groups I, II, and III based on resistance to antimicrobial agents and plasmid profiles. Each group expresses a characteristic level of resistance to gentamicin, tobramycin, and sisomicin. Enzyme assays showed that resistant strains expressed 2″-aminoglycoside phosphotransferase–6′-aminoglycoside transferase activity by a determinant which is known to be chromosomally located. The gentamicin resistance (Gm^r) determinants were transferred from group I, II, or III strains by transduction into a laboratory strain where each expressed the same low level of resistance. This finding suggests that high-level resistance in some clinical strains is due to a second, unlinked resistance mechanism. No evidence was obtained by hybridization experiments that clinical isolates or spontaneous mutants expressing high-level Gm^r carried more than one copy of the Gm^r determinant, thus eliminating the possibility that a gene dosage effect was responsible for high-level resistance. Hybridization experiments with transductants and wild strains suggested that the Gm^r determinant was located at homologous sites in wild strains from different groups, although restriction site differences were observed in flanking sequences. Electron microscope analysis of a cloned Gm^r determinant and genetic evidence suggested that a Dublin clinical isolate harbored a transposon very similar to Tn*4001*.

Methicillin- and gentamicin-resistant strains of *Staphylococcus aureus* (MGRSA) constitute a major problem in outbreaks of nosocomial infections worldwide (3, 8, 10, 14). In *S. aureus*, resistance to the aminoglycoside antibiotic gentamicin (Gm^r) and coincident resistance to kanamycin and tobramycin is due to the production of a bifunctional 2″-aminoglycoside phosphotransferase–6′-aminoglycoside [APH(2″)-AAC(6′)] modifying enzyme (27). The genetic determinant responsible for this resistance phenotype may be located on the plasmid or chromosome (8, 10, 14, 17, 33, 35). Plasmid elimination experiments showed that in Dublin MGRSA strains the determinant is invariably chromosomally encoded (8). In United States isolates the determinant is found on large, structurally related conjugative plasmids (12). In the majority of Australian isolates the determinant is also plasmid encoded (14, 33). However, Australian Gm^r plasmids are unrelated to their United States counterparts. They are nonconjugative and belong to the IncI incompatibility group (18). A 4.7-kilobase (kb) transposon, Tn*4001*, was found on a plasmid specifying gentamicin resistance in Australia (19). In addition, sequences homologous to that of Tn*4001* have been found on aminoglycoside resistance plasmids isolated from United States strains. However, these Gm^r determinants have inverted repeats that are 0.75 kb shorter than those of Tn*4001* and are not transposable (18).

MGRSA strains isolated in Dublin hospitals before 1985 were classified into two main groups based on resistance to a range of antimicrobial agents and on plasmid profiles (8). In addition, the two groups expressed different levels of resistance to gentamicin (MIC, 100 μg/ml in group I and 10 μg/ml in group II). Atypical group II strains exhibited the same antimicrobial resistance pattern and plasmid profile as group II strains, but the level of Gm^r was higher and ranged from 80 to 5,000 μg/ml. More recently it has been shown that a new MGRSA strain (group III), most probably introduced from Iraq, has become prevalent in Dublin hospitals (D. Carroll, H. Pomeroy, C. Keane, R. Russell, and D. Coleman, First International Conference of the Hospital Infection Society, abstr. 09/7, 1987).

We report here molecular cloning and expression of Gm^r determinants from several Dublin MGRSA strains in *Escherichia coli* and *S. aureus*. Evidence is presented that a Dublin MGRSA strain contains a functional transposon similar to Tn*4001*. Southern blot hybridization analyses were used to compare Irish and foreign isolates and showed that group I, II, and III MGRSA strains carry a single copy of the APH(2″)-AAC(6′) determinant chromosomally.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strain C600 (1) was used as a recipient in cloning experiments. *S. aureus* RN4220 (16), a restriction-deficient derivative of strain 8325-4, was used as a recipient in *S. aureus* cloning and transduction experiments.

Clinical isolates. MGRSA strains were obtained from the Federated Dublin Voluntary Hospitals, the Mater Misericordiae Hospital, and Dr. Steeven's Hospital, Dublin (Table 1).

Plasmids. *E. coli* and *S. aureus* cloning vectors and chimeric plasmids are listed in Table 2.

Bacteriological media and culture conditions. *E. coli* strains were grown in L broth (LB) or on L agar (LA) (21). *S. aureus* strains were cultured in tryptic soy broth (TSB) or tryptic soy agar (TSA) (Oxoid Ltd., Basingstoke, United Kingdom). Antibiotic susceptibility tests were performed on diagnostic sensitivity agar (DST; Oxoid). MIC determinations were

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TABLE 1. Bacterial strains

Strain	Characteristics	Reference or source
SA240, SA9, SA16	Group I MGRSA isolates from Dublin, 1976–1977	8
SA240MA, SA24MB	Spontaneous high-level Gm^r mutants of SA240	This study
SA240TD	RN4220 transduced with the Gm^r from SA240	This study
SA79, SA8, SA674	Group II MGRSA isolates from Dublin, 1978–1984	8
SA79A, SA79B	Spontaneous high-level Gm^r mutants of SAF49	This study
SA79TD	RN4220 transduced with the Gm^r from SA79	This study
SAF49, SA78, SA765, SA279P	Atypical group II MGRSA isolates from Dublin 1984–1985	8
SAF49A, SAF49B	Spontaneous high-level Gm^r mutants of SAF49	This study
SA78TD	RN4220 transduced with the Gm^r from SA78	This study
SA21, SA34, SA690	Group III MGRSA isolates from Dublin, 1985–1986	D. Carroll (Department of Microbiology, Trinity College)
SA21TD	RN4220 transduced with the Gm^r from SA21	This study
WG523	Australian isolate with chromosomal Gm^r	33
WG1320	RN450 carrying pWG53	33
Wilson	U.S. MGRSA isolate with conjugative Gm^r plasmid	S. Cohen (University of Chicago, Chicago, Ill.)
La Croix	U.S. MGRSA isolate with conjugative Gm^r plasmid	S. Cohen
Nicholls	U.S. MGRSA isolate with nontransferable Gm^r plasmid	S. Cohen
SA50	MGRSA strain from United Kingdom with plasmid-encoded Gm^r	M. De Saxe (Public Health Laboratory Service, Colindale, London)
M353(pTU068)	Japanese plasmid-encoded Gm^r	M. Konno (Teikyo University, Tokyo, Japan)
RN2677(pG02)	Conjugative Gm^r plasmid	2
A118(pA118)	Conjugative Gm^r plasmid	22
MS001-MS005	*S. aureus* RN4220 transposed with the Gm^r determinant from plasmid pDU1378	This study
RN4220	Mutant of 8325-4 (reference 23) capable of accepting shuttle plasmids from *E. coli*	16

carried out on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.).

Antimicrobial agents and susceptibility testing. A disk diffusion method (29) was used to test susceptibility to the following aminoglycosides: gentamicin (15 μg), kanamycin (30 IU), tobramycin (10 μg), amikacin (30 μg), sisomicin (10 μg), netilmicin (30 μg), and neomycin (30 IU). Antibiotic disks were purchased from Diagnostics Pasteur, Marne, France.

MIC determinations. Narrow-interval MICs were determined as described previously (7).

Bacteriophage typing. Bacteriophage typing was performed as described previously (6).

Assays for aminoglycoside-modifying enzymes. Extracts of *S. aureus* strains were prepared as previously described (9). The enzyme assays were performed by the method of Haas and Dowding (13).

Transduction. The Gm^r determinant was transduced from *S. aureus* clinical isolates SA79, SA78, and SA21 into *S. aureus* RN4220 by the method of Asheshov (4) with experimental phages C6, C7, and 932, respectively, kindly donated by H. Pomeroy, Department of Microbiology, Trinity

TABLE 2. Plasmids

Plasmid	Resistance markers	Relevant characteristics	Reference or source
pBR322	Ap^r Tc^r	*E. coli* cloning vector	5
pCW59	Cm^r Tc^r	*S. aureus* cloning vector	36
pC221	Cm^r	*S. aureus* cloning vector	24
pDU1370	Ap^r Gm^r Km^r Tm^r	pBR322 Ω (SA240 *Eco*RI 9.5 kb)	This study
pDU1371	Ap^r Gm^r Km^r Tm^r	pBR322 Ω (SA79 *Eco*RI 10 kb)	This study
pDU1372	Ap^r Gm^r Km^r Tm^r	pBR322 Ω (pDU1370 *Hin*dIII 2.6 kb)	This study
pDU1373	Ap^r Gm^r Km^r Tm^r	pBR322 Ω (pDU1371 *Hin*dIII 2.6 kb)	This study
pDU1374	Cm^r Gm^r Km^r Tm^r	pCW59 Ω (pDU1372 *Hin*dIII 2.6 kb) orientation 1	This study
pDU1375	Cm^r Gm^r Km^r Tm^r	pCW59 Ω (pDU1372 *Hin*dIII 2.6 kb) orientation 2	This study
pDU1376	Cm^r Gm^r Km^r Tm^r	pCW59 Ω (pDU1373 *Hin*dIII 2.6 kb) orientation 1	This study
pDU1377	Cm^r Gm^r Km^r Tm^r	pCW59 Ω (pDU1373 *Hin*dIII 2.6 kb) orientation 2	This study
pDU1378	Ap^r Cm^r Gm^r Km^r Tm^r	pDU1370 linked to PC221 at *Eco*RI sites	This study
pSF915A	Ap^r Gm^r Km^r Tm^r	pUC9 Ω (pIP1800 *Alu*I 1.5 kb) cloned from *S. faecalis*	11

TABLE 3. MICs of aminoglycosides for Dublin MGRSA strains and *S. aureus* transductants

Strain	Phenotypic group	MIC (μg/ml)[a]					
		Gentamicin	Kanamycin	Tobramycin	Sisomicin	Amikacin	Netilmicin
SA240	I	80	400	20	20	5.0	2.0
SA9	I	80	400	20	20	5.0	2.0
SA16	I	80	400	40	20	5.0	2.0
SA240MA		3,000	>5,000	>200	>200	>40	20
SA240MB		>4,000	>5,000	>200	>200	>40	20
SA240TD		7	100	10	10	1.0	1.0
SA79	II	8	>1,000	4.0	2.0	1.0	0.4
SA8	II	8	>1,000	4.0	2.0	1.0	0.4
SA674	II	8	>1,000	4.0	2.0	1.0	0.4
SA79MA		20	100	5.0	5.0	1.0	0.4
SA79MB		20	100	5.0	5.0	1.0	0.4
SA79TD		7	100	3.0	10	1.0	1.0
SA78	Atypical II	>5,000	>5,000	>200	>200	>40	20
SA765	Ayptical II	200	>5000	80	100	20	10
SA229P	Atypical II	1,600	>5,000	>200	200	40	20
SAF49	Atypical II	100	>5,000	60	100	6.0	10
SAF49MA		1,000	>5,000	>200	>200	>40	15
SAF49MB		>4,000	>5,000	>200	>200	>40	20
SA78TD		7.0	100	3.0	10	1.0	1.0
SA21	III	60	>5,000	20	10	5.0	1.0
SA34	III	60	>5,000	20	10	5.0	1.0
SA690	III	60	>5,000	20	10	1.0	1.0
SA21TD		7.0	100	3.0	10	1.0	1.0
RN4220		0.2	1.0	0.2	0.2	0.2	0.2

[a] Concentrations of aminoglycoside antibiotics for MIC determinations were used at the following intervals: 0 to 1.0 μg/ml, 0.2-μg/ml intervals; 2.0 to 10 μg/ml, 2-μg/ml intervals; 10 to 100 μg/ml, 10-μg/ml intervals; 100 to 1,000 μg/ml, 100-μg/ml intervals; 1,000 to 5,000 μg/ml, 100-μg/ml intervals.

College, Dublin. The determinant was transduced from the clinical isolate SA240 into *S. aureus* RN1030 by using phage 85 and subsequently into RN4220 by using phage 80. Transductants were selected on TSA supplemented with gentamicin (10 μg/ml).

Molecular cloning. Standard procedures for DNA isolation and molecular cloning were employed (20). Restriction endonucleases, T4 DNA ligase, DNase I, and DNA polymerase I were purchased from the Boehringer Corporation Ltd., Dublin, and were used according to the manufacturer's instructions.

Molecular cloning in pBR322. Purified total DNA (1 μg) from *S. aureus* SA240 and SA79 was cleaved with *Eco*RI, mixed with *Eco*RI-cleaved pBR322, ligated, and transformed into *E. coli* C600. Transformants were selected on agar supplemented with ampicillin (100 μg/ml) and replica plated onto LA supplemented with kanamycin (10 μg/ml) and neomycin (10 μg/ml).

Cloning in *S. aureus*. *Hin*dIII-cleaved pDU1372 and pDU1373 DNA were ligated to *Hin*dIII-cleaved pCW59 DNA. The Tc^r determinant of pCW59 is located on a *Hin*dIII fragment and was replaced by the Gm^r determinants cloned from pDU1372 or pDU1373. Protoplasts of strain RN4220 were transformed with ligated DNA as described previously (25), selecting for resistance to chloramphenicol (4 μg/ml). Transformants were replica plated onto agar plates supplemented with gentamicin (10 μg/ml). The orientation of the Gm^r gene with respect to vector sequences was determined by cleavage with *Nci*I, which cuts asymmetrically within the insert.

Isolation of high-level resistant mutants. Overnight cultures of SA240, SA79, and SAF49 were diluted and plated onto TSA supplemented with gentamicin (100 to 5,000 μg/ml).

DNA hybridization. DNA probes were labeled in vitro by nick translation (26) with [^{32}P]dATP (New England Nuclear Corp.). Total cellular DNA was cleaved with *Eco*RI or *Hin*dIII, fractionated in 1% agarose gels, and transferred to nitrocellulose sheets (Schleicher & Schuell Inc., Keene, N.H.) by the method of Southern (28).

Construction of pDU1378. A shuttle plasmid was constructed by joining plasmid pC221 to *Eco*RI-linearized pDU1370 and transformed into *E. coli* C600, selecting for Ap^r Km^r Cm^r. Protoplasts of RN4220 were transformed with pDU1378; transformants were selected on chloramphenicol (4 μg/ml) and replica plated onto TSA supplemented with gentamicin (10 μg/ml) and ampicillin (100 μg/ml).

Transposition experiments. *S. aureus* RN4220 carrying pDU1378 was cultured for 21 generations in TSB at 43°C. Dilutions were plated on TSA supplemented with gentamicin (10 μg/ml) and replica plated onto plates containing chloramphenicol (10 μg/ml) at 37°C. Gm^r Cm^s isolates were purified and screened for the absence of plasmid DNA (30).

Electron microscopy. Homoduplexes were constructed by using 0.5 μg of *Eco*RI-cleaved pDU1370 DNA which was denatured in a solution of 70% formamide–0.3 M NaCl–10 mM Tris–1 mM EDTA (pH 8.5) heated to 75°C for 5 min. The samples were allowed to renature at room temperature for 2 h. Specimens were mounted on celloidin-coated films supported by 200-mesh copper grids by the method of Kleinschmidt (15). After staining with phosphotungstic acid, the grids were examined in a Hitachi H 7000 scanning transmission electron microscope at a magnification of ×10,000. Plasmid pCW59 (36) DNA was included as a size standard.

RESULTS

Resistance to aminoglycosides expressed by Dublin MGRSA strains. The MIC of aminoglycosides for Dublin MGRSA are given in Table 3. These values confirm a previous report (8) indicating that Dublin MGRSA strains can be classified according to resistance phenotype. Strains SA21, SA34, and

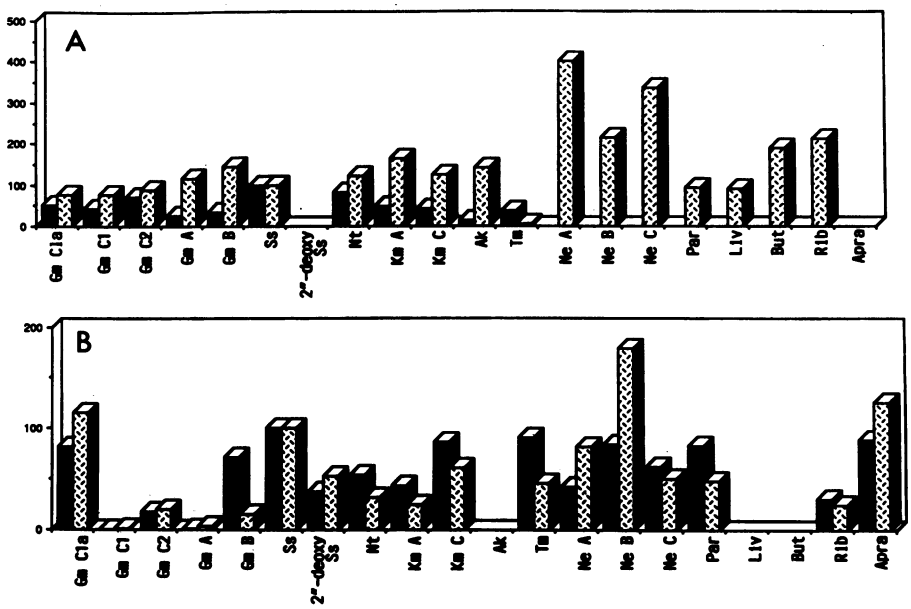

FIG. 1. Substrate profiles of enzymes extracted from SA24 (A) and SA79 (B). Phosphorylation (■) and acetylation (▨) are expressed relative to sisomicin or neomycin B as 100%. Aminoglycosides are abbreviated as follows: Gm, gentamicin; Ss, sisomycin; Nt, netilmicin; Km, kanamycin; Ak, amikacin; Tm, tobramycin; Ne, neomycin; Par, parcomycin; Liv, lividomycin; But, butirosin; Rib, ribostomycin; Apra, apramycin.

SA690, which were isolated more recently and were probably introduced by a patient traveling from Iraq, have intermediate levels of resistance to aminoglycosides compared with the group I and group II strains (Table 3). Group II, atypical group II, and group III strains harbored a plasmid-encoded Km^r Ne^r determinant which accounts for the high level kanamycin resistance in these strains.

Aminoglycoside-modifying activity of clinical isolates. The substrate profiles of aminoglycoside-modifying enzymes expressed by strains SA240 and SA79 are shown in Fig. 1. The acetylating and phosphorylating activities of both strains were characteristic of an APH(2″)-AAC(6′) bifunctional enzyme. The neomycin-modifying enzyme of strain SA79 was identified as a 3′-*O*-aminoglycoside phosphotransferase type III mechanism and was confirmed to be responsible for Km^r Ne^r in group II, atypical group II, and group III strains by hybridization to a gene-specific probe (34) (unpublished results).

Cloning gentamicin resistance determinants. The Gm^r determinants from SA240 (group I) and SA79 (group II) were cloned in pBR322 on *Eco*RI fragments, forming plasmids pDU1370 and pDU1371, respectively. Both determinants were subsequently subcloned on 2.6-kb *Hin*dIII fragments in pBR322, forming plasmids pDU1372 and pDU1373, respectively. The Gm^r determinants expressed low levels of resistance to the range of aminoglycosides modified by *S. aureus* extracts (Table 4). Southern hybridization was performed with *Hin*dIII-cut pDU1372 and pDU1373 DNA by using a 1.5-kb *Alu*I fragment carrying the Gm^r determinant cloned from *Streptococcus faecalis*. A 2.6-kb *Hin*dIII fragment hybridized, confirming that the APH(2″)-AAC(6′) determinant had been cloned in both cases (unpublished data).

Expression of the cloned gentamicin resistance determinants in *S. aureus*. The Gm^r determinants from pDU1372 and pDU1373 were subcloned in the *S. aureus* plasmid pCW59, forming pDU1374 and pDU1376, respectively. In strain RN4220 the cloned determinant from a phenotypic group I strain expressed the same level of resistance as the cloned phenotypic group II determinant (Table 4). The level was intermediate between those expressed by the parental group I and group II strains (Table 4). The level of expression also appeared to be dependent to some extent on the orientation of the cloned DNA with respect to vector sequences (Table 4).

Southern hybridization analysis of Gm^r determinants from clinical isolates. Genomic DNA from MGRSA strains isolated in Dublin, in which Gm^r is known to be chromosomally located (8), was cleaved with *Eco*RI, an enzyme which does not cut within the prototype Gm^r transposon Tn*4001* (19). Southern hybridization was performed with pSF915A as a probe (Fig. 2). In each case, a single fragment hybridized, suggesting that a single copy of the determinant was present. Similar results were obtained when blots were performed with DNA cleaved with *Eco*RV, another enzyme which cuts outside the transposon. In addition, the size of the hybridizing fragment was different in group I, II, and III strains. In group I, typical group II, and group III strains the size of the hybridizing fragment was the same for every member of the class tested. In contrast, each of the atypical group II strains tested had a different-sized hybridizing *Eco*RI fragment, implying a different chromosomal location of the Gm^r determinant in each strain.

DNA from Dublin MGRSA strains and isolates from other parts of the world were cut with *Hin*dIII, an enzyme which cuts within the inverted repeats of Tn*4001* (19). In each strain tested, a 2.6-kb fragment hybridized (Fig. 3).

Transduction of gentamicin resistance. Gm^r determinants from strains SA240, SA79, SA78, and SA21 were transduced into strain RN4220. The transductants were shown by DNA hybridization to have acquired APH(2″)-AAC(6′) sequences. Interestingly, transductants from each strain expressed the same low level of aminoglycoside resistance in RN4220 (Table 3). Two transductants from each MGRSA donor strain were analyzed by Southern hybridization when *Eco*RV-cleaved chromosomal DNA was probed with pSF 915A. Each transductant was shown to have the Gm^r deter-

TABLE 4. MICs of aminoglycosides for *S. aureus* and *E. coli* clones and transposon insertions

Strain or insertion	MIC (μg/ml)[a]					
	Gentamicin	Kanamycin	Tobramycin	Sisomicin	Amikacin	Netilmicin
E. coli						
C600	0.2	0.4	0.6	0.2	0.2	0.2
C600(pDU1372)	2.0	4.0	2.0	0.4	0.5	0.2
C600(pDU1373)	2.0	4.0	2.0	0.4	0.5	0.2
S. aureus						
RN4220	1.0	0.5	1.0	0.2	0.2	0.2
SA240	80	>1,000	20	20	5	2
RN4220(pDU1374, orientation 1)	80	100	10	10	1.0	1.0
RN4220(pDU1375, orientation 2)	40	40	5	4	0.5	0.4
SA79	8.0	>1,000	4.0	2.0	1.0	0.4
RN4220(pDU1376, orientation 1)	80	100	10	10	1.0	1.0
RN4220(pDU1377, orientation 2)	40	40	5	4	0.5	0.4
Transposon insertions						
RN4220(pDU1378)	20	20	5.0	10	1.0	
MS001	20	40	5.0	10	1.0	
MS002	6.0	10	3.0	5.0	0.8	
MS003	10	20	5.0	8.0	1.0	
MS004	9.0	20	4.0	8.0	0.8	
MS005	7.0	20	3.0	5.0	0.8	

[a] See footnote *a* of Table 3.

minant located on the same-sized restriction fragment (data not shown), whereas in the donor strains the marker was associated with different restriction fragments, the sizes of which were characteristic of the MGRSA group (Fig. 2).

Gm^r transposon is present in a Dublin MGRSA strain. A shuttle plasmid (pDU1378) was constructed which carried a 9.5-kb *Eco*RI fragment encoding Gm^r isolated from strain SA240. If the Gm^r determinant of strain SA240 is located on a transposon similar to Tn*4001*, it should be present intact in pDU1378 because Tn*4001* does not have any *Eco*RI sites. Plasmid pDU1378 was transferred into *S. aureus* RN4220, and the plasmid was then cured from a population of cells maintained by serial dilution in broth incubated at 43°C.

Five Gm^r isolates which had lost Cm^r, the marker linked to the replication origin of the vector pDU1378, were shown to lack detectable plasmid DNA. MIC determinations showed that resistance to the range of aminoglycoside antibiotics modified by the APH(2″)-AAC(6′) bifunctional enzyme was expressed by these derivatives, but the level differed in each strain (Table 4).

To provide direct evidence for transposition of the Gm^r determinant into the RN4220 chromosome, genomic DNA of the Gm^r Cm^s strains was cut with *Cla*I. Restriction mapping experiments have shown that this enzyme cleaves within the inverted repeats of Tn*4001*-like elements (unpublished data). The *Cla*I-cleaved DNA was probed with nick-translated pDU1370 DNA. In each isolate two unique *Cla*I fragments were seen among the bands which hybridized (data not

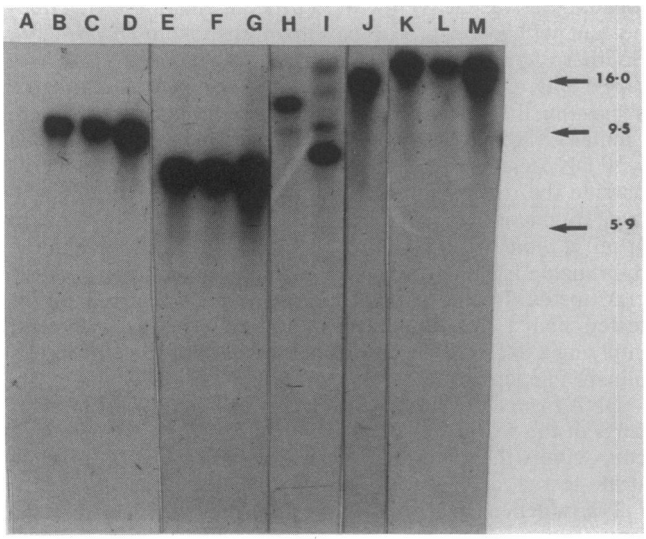

FIG. 2. Autoradiogram of Southern blot hybridization of *Eco*RI-cut total cellular DNA isolated from Dublin MGRSA strains probed with pSF915A, a pUC9 derivative containing a 1.5-kb DNA sequence encoding Gm^r isolated from *S. faecalis*. Tracks: A, RN4220; B, SA240; C, SA9; D, SA16; E, SA79; F, SA8; G, SA674; H, SAF49; I, SA78; J, SA765; K, SA21; L, SA34; M, SA690. Other hybridization experiments (data not shown) confirmed that the multiple bands hybridizing to the probe in track I were due to partial digestion of the DNA.

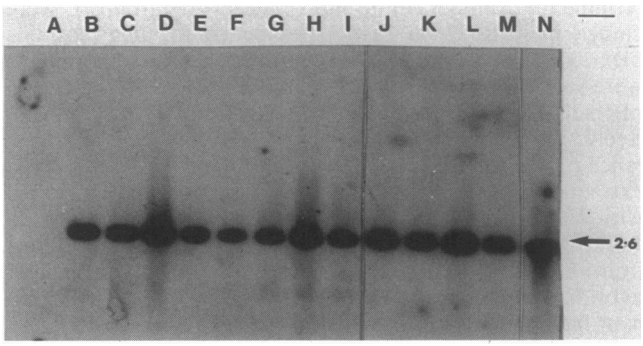

FIG. 3. Autoradiogram of Southern blot hybridization of *Hin*dIII-cut total cell DNA isolated from Dublin and foreign MGRSA strains probed with pSF915A. Tracks: A, RN4220; B, SA240; C, SA79; D, SA21; E, SA78; F, MS353(pTU068); G, WG1320; H, WG523; I, Wilson; J, Nicholls; K, La Croix; L, 8325-4(pG02); M, A118(pA118); N, SA50.

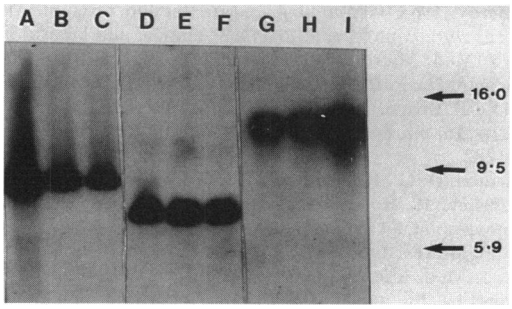

FIG. 4. Autoradiogram of *Eco*RI-cut total cellular DNA from Dublin group I, group II, and atypical group II strains and high level resistant mutants probed with pSF915A. Tracks: A, SA240; B, SA240MA; C, SA240MB; D, SA79; E, SA79MA; F, SA79MB; G, SAF49; H, SAF49MA; I, SAF49MB.

shown). These are probably junction fragments between the putative Gm^r transposon and chromosomal sequences. The fact that different fragments were seen with each strain is consistent with Gm^r being introduced into the chromosome by transposition. Furthermore, the hybridization pattern of parental strain RN4220 remained unchanged in each of the isolates, suggesting that transposition had occurred elsewhere in the chromosome.

Isolation and characterization of Gm^r mutants. Spontaneous mutants expressing higher levels of resistance to gentamicin were isolated from wild-type group I, group II, and atypical group II MGRSA strains. In the case of the mutants of SA240 and SAF49 there was a stable concomitant increase in resistance to other aminoglycosides modified by the APH(2″)-AAC(6′) determinant (Table 3). Southern hybridization analysis of genomic DNA from mutant strains cleaved with *Eco*RI (Fig. 4) and *Hin*dIII showed that no gross rearrangement of the determinant was associated with increased resistance.

Electron microscope analysis of pDU1370 DNA. DNA of plasmid pDU1370 was cleaved with *Eco*RI, denatured, and reannealed. Intramolecular hybridization of single-stranded DNA sequences could occur if they carry inverted repeat sequences. Figure 5 shows an example of the stem-loop structure having a 1.3-kb double-stranded stem and a 1.8-kb single-stranded loop similar in size to that reported for Tn*4001* (19).

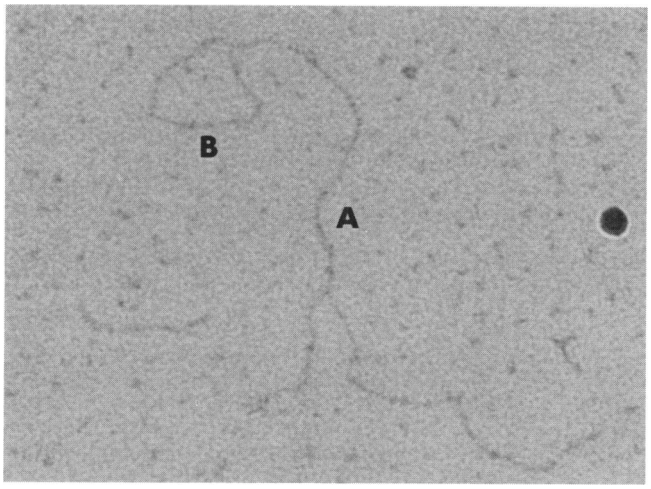

FIG. 5. Homoduplex of *Eco*RI-cleaved pSH01 DNA. The double-stem (A) and single-stranded loop (B) structures are indicated.

DISCUSSION

In this paper we demonstrate by enzyme assays with a range of aminoglycoside substrates and by DNA hybridization that a bifunctional APH(2″)-AAC(6′) mechanism is responsible for resistance to gentamicin, sisomicin, and tobramycin in Dublin MGRSA strains. Strains expressing Ne^r harbor an additional *aphA-3* determinant.

In Australian MGRSA strains Gm^r is usually plasmid encoded. Two groups have shown that this determinant is transposable (19, 32). Indeed, the same determinant is sometimes chromosomally located, presumably because of transposition from the Gm^r plasmid to the chromosome. The data described here provide good genetic and physical evidence that a Tn*4001*-like transposon is present in the chromosome of a typical group I Dublin MGRSA strain. Hybridization experiments showed that in each strain examined, including the strain carrying pWG53 which is known to carry a transposon that is similar if not identical to Tn*4001* (31), a 2.6-kb fragment hybridized to the intragenic probe. Gm^r transposed from a shuttle plasmid carrying the cloned Gm^r determinant into different sites on the *S. aureus* chromosome. Also, electron microscopic analysis revealed intramolecular base pairing, forming stem-loop structures similar in dimension to Tn*4001*. It is also shown that group II and III strains have a similar transposon in the chromosome but at different sites. It is reasonable to suppose that dissemination of the Gm^r determinant by transposition has contributed to the spread of Gm^r in *S. aureus* and *Staphylococcus epidermidis*. However, the determinants associated with coagulase-negative staphylococci and *S. aureus* isolates from American hospitals are found on large conjugative plasmids, have shorter inverted repeats, and are nontransposable (18).

This study set out to provide an explanation for high levels of resistance to aminoglycosides expressed by group I and atypical group II Dublin MGRSA strains and higher-level resistant mutants derived from them. Transfer and hybridization experiments eliminate the following possible explanations. (i) Gene amplification by tandem duplication is ruled out because the elevated resistance is stably inherited in the absence of selection (unpublished data). Tandem repeats would be unstable and would be expected to revert rapidly to the monomeric state. No evidence for multiple gene copies was found in hybridization experiments. (ii) Increased transcription or translation of mRNA of the APH(2″)-AAC(6′) gene would be a *cis*-acting phenomenon linked to the determinant in transduction, transposition, or cloning experiments. (iii) Acquisition of multiple copies of the Gm^r marker by sequential transposition is also eliminated by hybridization of genomic DNA cleaved with *Eco*RI or *Eco*RV, enzymes which do not cut within Tn*4001*. If more than one copy of the transposon were present, more than one fragment would be expected to hybridize. (iv) Increasing gene dosage is probably not an efficient mechanism of increasing resistance, judging by the 10-fold increase in resistance expressed by the cloned Gm^r determinant from SA240 linked to pCW59, a plasmid which has a copy number of approximately 120 (35).

By a process of elimination it can only be inferred that group I, atypical group II, and group III strains have an additional, unlinked resistance mechanism(s) which acts synergistically with the APH(2″)-AAC(6′) enzymatic mech-

anism. Thus each phenotypic group had the Gm^r marker in different-sized restriction fragments which were characteristic of the group concerned. Atypical group II strains were the exception; each strain tested had a different-sized hybridizing fragment.

We have tried to seek an explanation for the vastly different levels of resistance expressed by Dublin MGRSA strains. Each type appears to have a single copy of the APH(2″)-AAC(6′) determinant in the chromosome. When this determinant was transferred by transduction into a laboratory strain of *S. aureus*, resistance was expressed at the same low level irrespective of the origin of the marker. Similarly, the cloned Gm^r determinants from group I and II strains also expressed indistinguishable levels of resistance when introduced into RN4220 on the same vector. This shows that the genetic determinant of elevated Gm^r is not linked to the transposon and suggests that a second, unliked mechanism which acts synergistically with the enzymatic mechanism is present in clinical isolates exhibiting high levels of resistance.

It is possible that mutations affecting ribosomal structure or drug transport have been selected. Unfortunately, since the APH(2″)-AAC(6′) determinants were chromosomal and not plasmid located, it was not possible to study wild-type isolates from which the determinant had been lost.

The five derivatives of RN4220 which had the transposon from strain SA240 inserted at different chromosomal sites expressed resistance to aminoglycosides which varied over a threefold range. However, when the Gm^r determinants were transduced from group I and II, atypical group II, and group III donors into a common recipient strain, they were associated with identically sized *Eco*RV restriction fragments (unpublished data). This suggests that the determinants are located at sites that are basically homologous in the different donor strains but which have acquired restriction site polymorphisms. If this is so, then MGRSA strains which have chromosomally located Gm^r determinants may be derived from the same ancestral strain rather than having evolved at different times by independent transposition events.

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