# GENTAMICIN AND METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS IN DUBLIN HOSPITALS: MOLECULAR STUDIES

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SUMMARY. A large number of *Staphylococcus aureus* strains resistant to gentamicin, methicillin and other antibiotics, isolated in several Dublin hospitals during a 4-year period, were screened for plasmid DNA. Isolates assigned to four principal phage groups showed uniform plasmid profiles. A plasmid of mol. wt $21 \cdot 0 \times 10^6$ encoding penicillin resistance was present in all isolates screened. A tetracycline resistance plasmid of mol. wt $24 \cdot 0 \times 10^6$ was present in 40% of isolates of phage types 90 and 5/47/54/84/85 whereas a plasmid of mol. wt $3 \cdot 0 \times 10^6$ was responsible for tetracycline resistance in isolates of phage types 77 and 85. Aminoglycoside, methicillin and erythromycin resistances were apparently not encoded on plasmids. Gentamicin resistance was transduced at low frequency between several strains of *S. aureus* and the resistance phenotype was due, in part at least, to the production of an inactivating enzyme.

## INTRODUCTION

Several recent reports have described the isolation of *Staphylococcus aureus* strains resistant to gentamicin, methicillin and other antibiotics. (Shanson, Kensit and Duke, 1976; Price, Brain and Dickson, 1980; Hone *et al.*, 1981). As with other bacteria, antibiotic resistance can be either chromosomally or plasmid encoded in *S. aureus* (Lacey, 1975; Shanson, 1981). However, unlike many plasmids found in gram-negative organisms, those in *S. aureus* do not appear to promote conjugative transfer of genetic material between strains. Transfer of antibiotic resistance determinants between *S. aureus* strains has been achieved in the laboratory using bacteriophages as vectors and similar transfer events could be responsible for the spread of antibiotic resistance between strains in the hospital environment (Novick *et al.*, 1979; Lacey and Lord, 1980).

At present it is not fully understood how antibiotic resistance determinants become established within *S. aureus* strains present in hospitals. Although genetic studies have been performed on antibiotic resistance genes in *S. aureus*, few attempts have been made to conduct a large survey by plasmid screening and simple genetic techniques to identify the mechanisms of antibiotic resistance present in large numbers

of related hospital isolates. Such a survey could be useful for collecting epidemiological information on the spread of multiple antibiotic resistance amongst *S. aureus* strains in hospitals.

Since 1976, gentamicin and methicillin resistant *S. aureus* (GMRSA) have been a major cause of infections in Dublin hospitals. Clinical studies have been conducted on these strains (Cafferkey *et al.*, 1983) which were shown to belong to a limited number of phage groups (Williams and Rippon, 1952). In parallel with clinical studies we have used simple molecular and genetic approaches to identify the genetic basis of antibiotic resistance in these strains and the results of the study are presented in this report.

## MATERIALS AND METHODS

*S. aureus strains, antibiotic susceptibility testing and phage typing.* All clinical isolates of *S. aureus* used in this study were isolated in the Federated Dublin Voluntary Hospitals (F.D.V.H.) or the Mater Misericordiae Hospital (M.M.H.), Dublin. The sources of the strains, antibiotic susceptibility testing and phage typing methods are described in detail in an accompanying paper (Cafferkey *et al.*, 1983). Strains for plasmid screening were stored in 40% (w/w) glycerol at −20°C. Recipient strain RN2677 and strains RN3612 and RN3499 that contained reference marker plasmids were obtained from Dr R. Novick (New York, USA). Strain RN3499 contained plasmids pI524 (mol. wt $21\cdot4 \times 10^6$), pT181 ($3\cdot0 \times 10^6$), pE194 ($2\cdot4 \times 10^6$) and pC194 ($1\cdot8 \times 10^6$); strain RN361 contained a plasmid of mol. wt $5\cdot3 \times 10^6$. Recipient strain SA1030 was described by Lacey and Lord (1980). Organisms were routinely cultured on Nutrient Agar or in Nutrient Broth (Oxoid).

*Rapid screening of isolates for plasmid DNA.* Isolates were screened by a modification of the technique of Hansen and Olsen (1978). Cells were harvested from 2 ml of an overnight nutrient broth culture using an Eppendorff Microfuge (model 5412). The pellet was washed once in 500 $\mu$l of 50 mM trishydroxylmethylaminomethane (Tris-HCl), 10 mM ethylenediaminetetra-acetic acid (EDTA) at $p$H $8\cdot0$ (TE buffer) and resuspended in 40 $\mu$l of TE buffer; 10 $\mu$l of lysotaphin (Sigma) 1 mg/ml in 50 mM Tris-HCl, $p$H $7\cdot5$, was then added. After incubation at 37°C for 10 min, 600 $\mu$l of freshly prepared lysing buffer (TE buffer containing 4% w/v sodium dodecyl sulphate (SDS), $p$H $12\cdot35$) was added and incubation was continued for a further 20 min to lyse the cells. The solution was neutralised with 30 $\mu$l of 2 M Tris-HCl, $p$H $7\cdot0$; 160 $\mu$l of 5 M NaCl was quickly added with mixing and the tube was left for 1 h on ice to precipitate chromosomal DNA. This appeared as a white floccular material and was removed by centrifugation for 15 min in the Microfuge. The supernate was decanted into a second microtube containing 550 $\mu$l of isoproponol; the tube was held at −20°C for at least 30 min then centrifuged for 5 min in the Microfuge. The supernate was discarded and the open tube was dried under vacuum. The dried precipitate was resuspended in 40 $\mu$l of TE buffer and either run in 20 $\mu$l portions through an agarose gel or stored at −20°C until required. Samples were electrophoresed in $0\cdot7\%$ (w/v) agarose gels at 70 V according to the method of Meyers *et al.* (1976). Up to 16 isolates could be screened at one time. Electrophoresis was continued until the blue tracker dye reached the end of the gel. The gels were stained with ethidium bromide solution and the DNA was visualised by ultra-violet illumination.

*Restriction enzyme analysis.* Plasmid DNA was cleaved with the restriction enzymes *Hind* III, *Eco*RI and *Bam* HI in conditions suggested by the manufacturers (New England Biolabs, CP Laboratories, Bishops Stortford, UK).

*Plasmid elimination experiments* were performed as described by O'Reilly *et al.* (1981). Strains were grown overnight in the presence of subinhibitory concentrations of ethidium bromide at 37°C or 43°C. Normally a final ethidium bromide concentration of 100–150 $\mu$l/ml was suitable.

*Transformation* of *S. aureus* strain RN2677 (Novick *et al.*, 1979) was performed by a modification of the protoplast method of Chang and Cohen (1979). A 50-$\mu$l inoculum of an overnight culture of recipient strain RN2677 (rifampicin and novobiocin resistant) was added to 50 ml of Penassay broth (Oxoid) and the culture was grown to an $OD_{600}$ of $0\cdot6$. Cells were

harvested in detergent-free sterile-capped plastic centrifuge tubes in a bench centrifuge, washed in SMMP buffer (doubly-concentrated SMM, see below, in four-fold concentrated Penassay broth; Chang and Cohen, 1979), and resuspended in 2·5 ml of SMMP; 100 $\mu$l of lysostaphin 1 mg/ml in 0·5 M sucrose, 0·02 M maleate, 0·02 M MgCl$_2$, pH 6·5 (SMM buffer) was added and the tubes incubated at 37°C for 30 min to generate protoplasts. The protoplasts were transferred to a second sterile centrifuge tube, diluted with an equal volume of SMMP, harvested by centrifugation, and resuspended in 2·5 ml of SMMP. The washed protoplasts were stable at room temperature for several hours.

For transformation experiments, 0·5 ml of protoplast suspension was mixed with 0·5 $\mu$g of plasmid DNA suspended in 100 $\mu$l of twice-concentrated SMM, then 1·5 ml of 40% (w/v) polyethylene glycol 6000 dissolved in twice-concentrated SMM was added with gentle mixing for 2 min. Five ml of SMMP was added and the protoplasts harvested immediately by centrifugation at maximum speed in a bench centrifuge. The supernate was removed and the tube allowed to drain for a few minutes. The pelleted protoplasts were resuspended in 1 ml of SMMP and left at 32°C for 4 h to allow expression of antibiotic resistance. Samples were then diluted and plated on to DM3 regeneration medium (Chang and Cohen, 1979) containing the selective antibiotic and rifampicin (1 $\mu$g/ml). Putative transformants appeared after 2 days and were purified on selective media, tested for resistance to rifampicin and novobiocin and screened for plasmid DNA. For this procedure to work reproducibly all media, glassware, pipettes and DNA samples had to be sterile and free of detergents that affect the stability of the protoplasts.

*Assay of aminoglycoside inactivating enzymes.* Assays were performed by the phosphocellulose binding technique of Benveniste and Davies (1973). Cells were harvested from 100-ml cultures grown overnight in Brain Heart Infusion broth (Difco) at 37°C and resuspended in 3 ml of 32 mM Tris-HCl, 16 mM MgCl$_2$, pH 7·6. The cells were treated with 200 $\mu$l of lysostaphin 1 mg/ml in 0·25 M Tris-HCl, pH 7·6 for 10 min at 37°C then broken by ultra sonication (MSE, model 7100) using six 30-s bursts with cooling on ice. Cell debris was removed by centrifugation at 48 000 $g$ for 30 min; the supernate was divided into 100-$\mu$l volumes and stored at 4°C until required. Assays were performed for acetyl, adenyl and phosphotransferase activity.

*Transduction* was performed by the method of Asheshov (1966). Phages 80 and 85 from the International Typing Set were employed to transduce resistance determinants to RN2677 and SA1030 (Lacey and Lord, 1980).

*Mixed culture experiments* were performed as described by Lacey (1971) using a rifampicin-resistant mutant of SA1030 as recipient.

RESULTS

*Screening of GMRSA for plasmids*

Several hundred clinical specimens of GMRSA have been collected since 1976 from infected patients in Dublin hospitals. Clinical studies described in an accompanying report (Cafferkey *et al.*, 1983) have shown that the isolates belong to a limited number of phage groups. The GMRSA strains were often resistant to several other antibiotics including kanamycin and erythromycin. As a preparation for detailed genetic studies, *c*. 200 GMRSA strains isolated at different times during the study period from each hospital were screened for the presence of plasmid DNA. A uniform plasmid profile was detected even though individual strains often exhibited slightly different antibiotic resistance phenotypes (fig. 1). Isolates of phage types 77 and 85 were the first GMRSA strains to cause infections in the hospitals (table). All representative strains of these isolates that were screened harboured a plasmid of mol. wt 21 $\times$ 10$^6$ (fig. 1, tracks E and F). In all, 40 isolates that had been isolated during a 2-year period from four F.D.V.H. hospitals were examined; 40% of these isolates harboured an additional plasmid of mol. wt 3 $\times$ 10$^6$ (fig. 1, tracks G and H) and were tetracycline resistant

TABLE

*Bacteriophage typing patterns and plasmid content of GMRSA isolates*

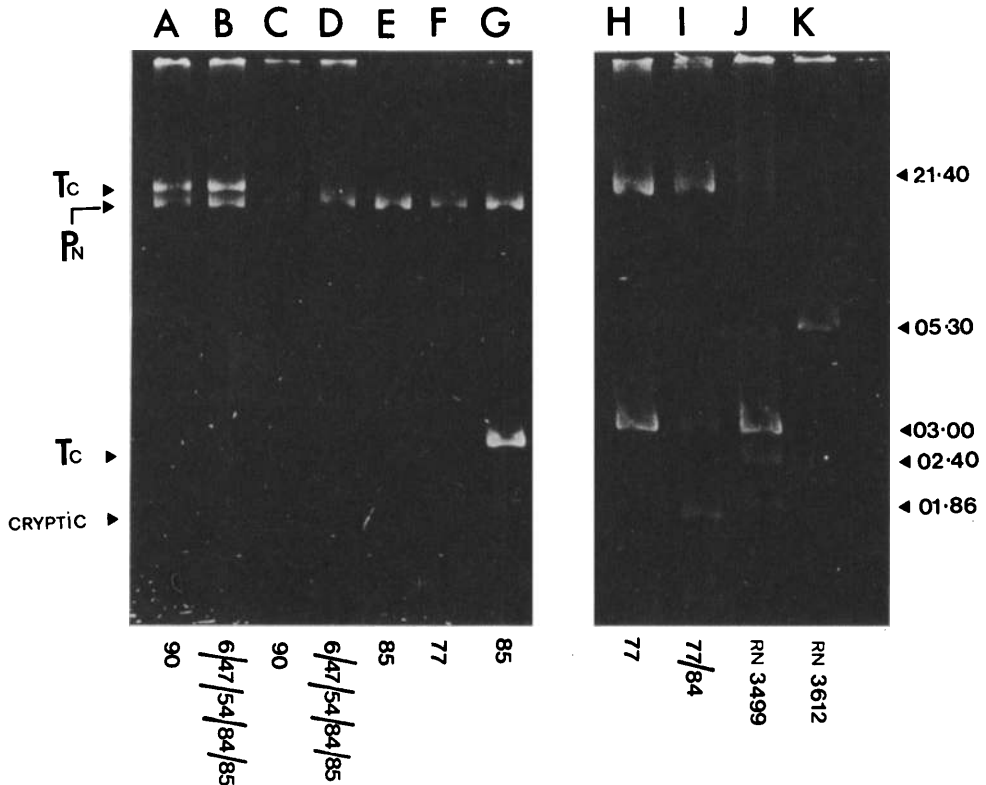| Phage type | Date of appearance in hospitals | Plasmids detected (mol. wt) | Number of isolates screened |
|---|---|---|---|
| | *F.D.V.H.* | | |
| 77/84 | 1977–1978 | $21 \times 10^6$ [1] | 21 |
| | | $3 \times 10^6$ [2] | |
| 85 | 1977/1979 | $21 \times 10^6$ | 18 |
| | | $3 \times 10^6$ | |
| 90 at 100 RTD | 1978–1980 | $24 \times 10^6$ [2] | 100 |
| | | $21 \times 10^6$ | |
| 6/47/54/84/85 | 1979 | $24 \times 10^6$ | 20 |
| | | $21 \times 10^6$ | |
| | *M.M.H.* | | |
| 90 at 100 RTD | 1979–1980 | $24 \times 10^6$ | 12 |
| | | $21 \times 10^6$ | |
| 77/85 | 1979 | $21 \times 10^6$ | |
| | | $1·7 \times 10^6$ | 10 |
| | | $1·3 \times 10^6$ | |

RTD = routine test dilution.



FIG. 1. Agarose gel electrophoresis of plasmid DNA isolated from GMRSA. The phage type of each isolate is indicated below tracks A–I. Tracks J and K contain plasmids of known mol. wt as controls (see *Methods*). Mol. wt ($\times 10^6$) is shown on the right. Tc = tetracycline resistance plasmid; Pn = penicillinase plasmid.

(Tc-R). None of the tetracycline sensitive (Tc-S) isolates harboured the $3 \times 10^6$ mol. wt plasmid (fig. 1, tracks E and F).

GMRSA strains that were untypable with standard phages but reacted with experimental phage 90 at 100 RTD appeared in the hospitals in 1977. These isolates quickly became the most common type of GMRSA isolated from infected patients. By early 1980 a small number of isolates of phage type 6/47/54/84/85 were isolated in one hospital. Plasmid screening again revealed a uniform plasmid profile for these strains and *c.* 40% of the isolates of both phage types were Tc-R. Such isolates harboured two plasmids of mol. wts $21 \times 10^6$ and $24 \times 10^6$ (fig. 1, tracks A and B). The remaining Tc-S isolates harboured only the $21 \times 10^6$ mol. wt plasmid (fig. 1, tracks C and D).

Sixteen GMRSA strains isolated in the M.M.H. group were screened. They were of phage type 77/84 or type 90 at 100 RTD. The type 77/85 isolates harboured plasmids of mol. wts $21 \times 10^6$, $1 \cdot 7 \times 10^6$ and $1 \cdot 3 \times 10^6$ (fig. 1, track I). Isolates of type 90 exhibited the same plasmid profiles as the F.D.V.H. type 90 isolates.

A large number of gentamicin sensitive (Gm-S) strains of *S. aureus* isolated in the same hospitals have been screened for plasmid DNA. The isolates belonged to various phage types. Several plasmids that were never detected in GMRSA isolates were found in these strains. Furthermore, some of these isolates harboured plasmids of mol. wts $21 \times 10^6$ and $3 \times 10^6$ (results not shown). Thus although other plasmids were present in *S. aureus* within the hospitals, with the exception of the $21 \times 10^6$ and $3 \cdot 0 \times 10^6$ mol. wt plasmids, none of these were found in GMRSA isolates.

### Restriction enzyme cleavage of plasmid DNA from GMRSA

Plasmid DNA purified from four isolates of each phage type was cleaved with the restriction enzyme *Eco*RI (fig. 2). Analysis of the restriction fragments on agarose gels revealed that the $21 \times 10^6$ mol. wt plasmids were closely related and probably identical in all strains. The $24 \cdot 0 \times 10^6$ mol. wt plasmid harboured by isolates of phage types 77 and 85 also gave rise to identical restriction fragments on digestion with *Eco*RI (fig. 2, tracks C and D). The $3 \cdot 0 \times 10^6$ mol. wt plasmid was cleaved once with *Eco*RI in all cases. Cleavage of plasmid DNA with *Bam*HI and *Hind*III also suggested that the plasmids of similar mol. wt were identical (not shown).

### Plasmid elimination experiments

Selected isolates belonging to each of the phage types were grown under conditions that favoured the loss of plasmid DNA. Individual colonies were then screened for loss of antibiotic resistance determinants. Growth in the presence of sub-inhibitory concentrations of ethidium bromide or novobiocin at 43°C failed to eliminate most antibiotic resistance determinants at a detectable frequency. Tc-R isolates of phage types 77 and 85 gave rise to Tc-S derivatives at a significant frequency. All Tc-S derivatives had lost the $3 \cdot 0 \times 10^6$ mol. wt plasmid. Tc-R isolates of type 90 or 5/47/54/84/85 lost penicillin resistance (Pn-R) at frequencies of up to 10% after overnight growth. All Pn-S derivatives had lost the $21 \cdot 0 \times 10^6$ mol. wt plasmid. Interestingly, Tc-S isolates of phage types 90 and 5/47/54/84/85 did not lose Pn-R at a detectable frequency.
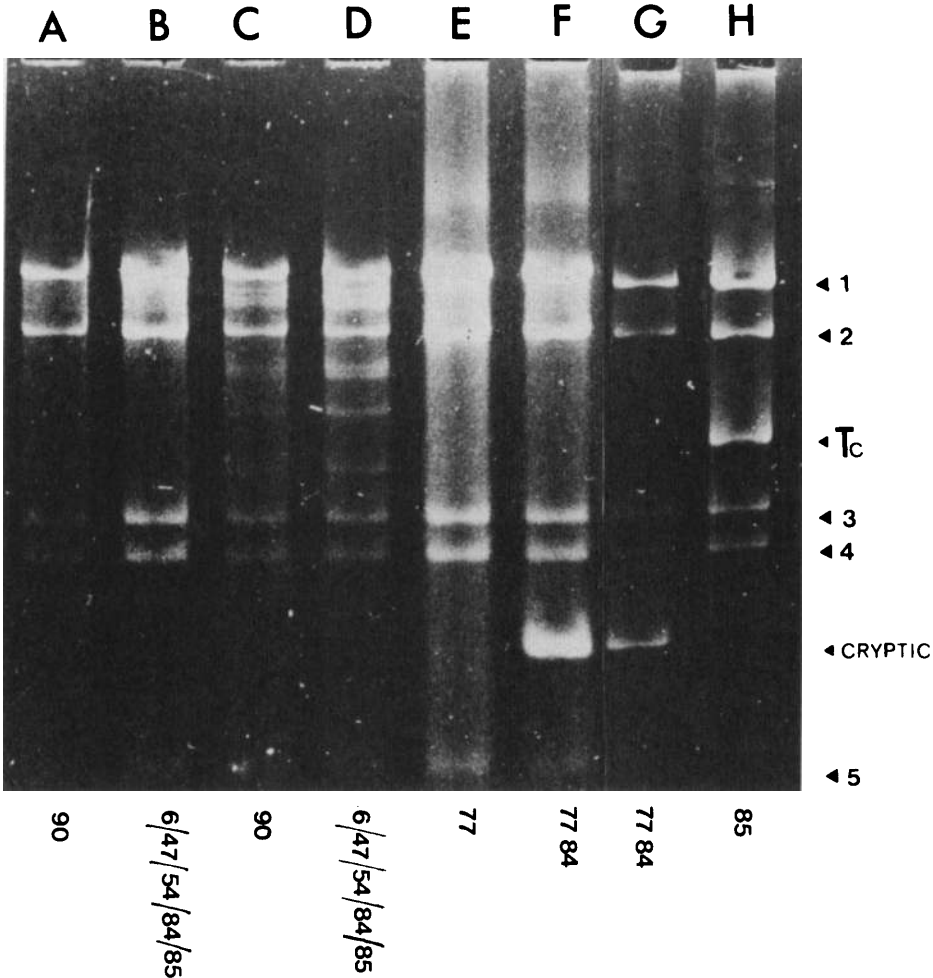
FIG. 2.   Agarose gel electrophoresis of purified plasmid DNA cleaved with restriction enzyme *Eco*RI.   The phage type of each isolate is indicated below each track.   Tc indicates the $3·0 \times 10^6$ mol. wt, Tc-R plasmid and "cryptic" refers to the $1·7 \times 10^6$ mol. wt plasmid found in type 77/84 isolates. Electrophoresis was on a 1% (w/v) agarose gel.

### Transfer of resistance determinants

Recipient strain RN2677 was transformed with plasmid DNA from each of the phage types.   Plasmid DNA isolated from Tc-R isolates of phage types 77 and 85 transformed RN2677 to Tc-R at high frequency (1 in $10^5$).   All Tc-R transformants harboured the $3·0 \times 10^6$ mol. wt plasmid (fig. 3, track D).   Pn-R transformants occurred at a lower frequency (1 in $10^7$) and all harboured the $21·0 \times 10^6$ mol. wt plasmid (fig. 3, track C).   Plasmid DNA prepared from isolates of phage types 90 or 5/47/54/84/85 gave identical results for Pn-R.   From these types, Tc-R transformants were obtained at a similar frequency to Pn-R and all Tc-R transformants harboured the $24·0 \times 10^6$ mol. wt plasmid.   RN2677 transformants resistant to gentamicin, amikacin, methicillin, kanamycin or erythromycin were not detected.
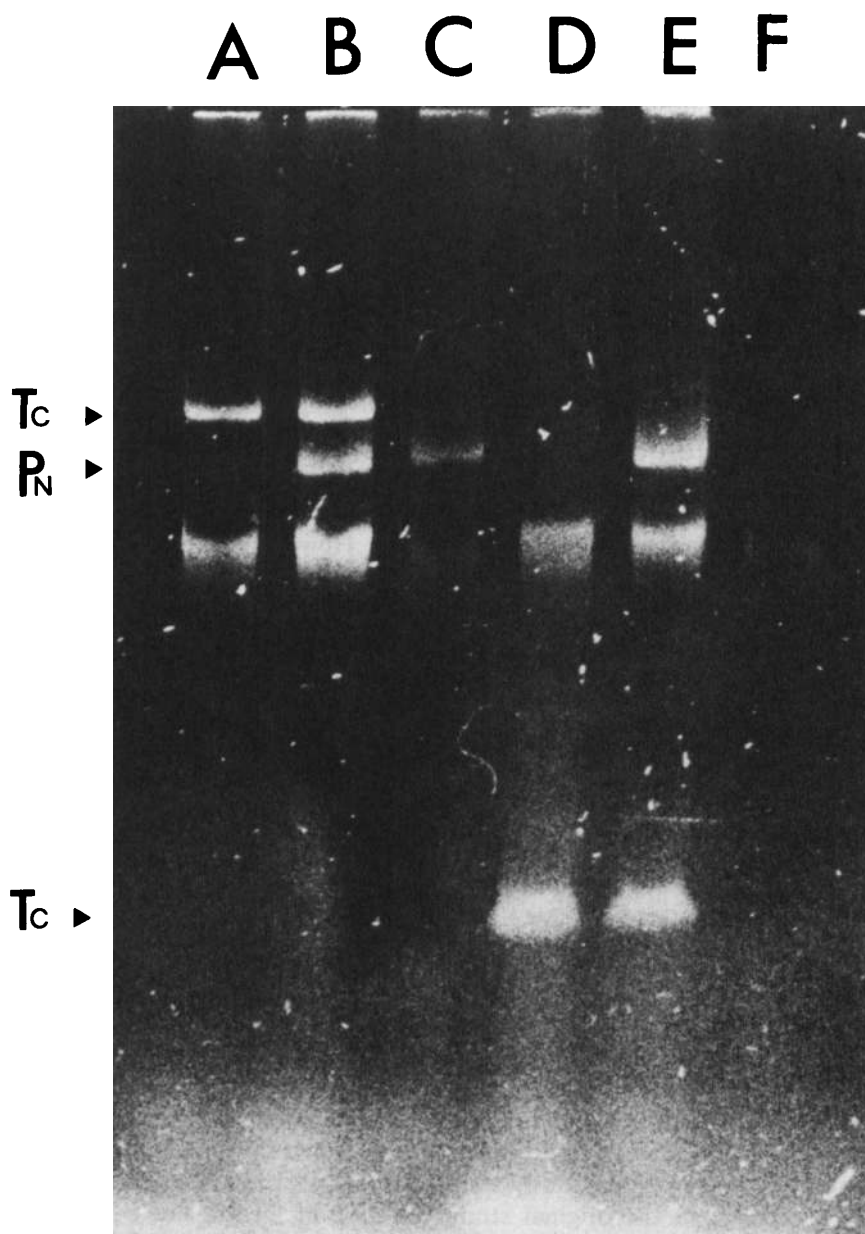
FIG. 3. Agarose gel electrophoresis of plasmid DNA isolated from RN2677 transformants. A, Tc-R transformant isolated after using type 90 DNA; B, type 90 DNA (control); C, Pn-R transformant isolated after using type 90 DNA; D, Tc-R transformant isolated after using type 85 DNA; E, type 85 DNA (control); F, Gm-R transductant of RN2677. Only chromosomal DNA can be seen in track F. Electrophoresis on different concentrations of agarose showed that no covalently closed circular plasmid DNA was obscured by this DNA.

Tc-R isolates of phage type 85 were used as donors in transduction experiments employing bacteriophage 85 from the phage typing collection. Neither Tc-R nor Gm-R was transferred to RN2677 or SA1030 (Lacey and Lord, 1980) in mixed culture experiments. Tc-R was readily transduced to these strains at frequencies up to $5 \times 10^{-5}$ and all transductants acquired the $3.0 \times 10^6$ mol. wt plasmid. Gm-R was transduced to both recipients at a much lower frequency of $(1-5) \times 10^{-8}$. Plasmid screening of Gm-R transductants failed to detect any new plasmid DNA. Gm-R could be transduced from SA1030 or RN2677 transductants by phage 80 at a similar frequency. No unselected antibiotic resistance was acquired by the transductants.

GMRSA and Gm-R transductants were assayed for aminoglycoside inactivating activity. Acetyltransferase and phosphotransferase activity was detected in all Gm-R strains of *S. aureus* but not in control strains.

## DISCUSSION

This report is part of an epidemiological survey currently being carried out on nosocomial infections involving *S. aureus* in a group of nine Dublin hospitals. Gentamicin sensitive *S. aureus* strains isolated in these hospitals belong to a wide range of different phage groups (unpublished results). However GMRSA strains belonged to a limited number of phage groups and possessed a remarkably uniform plasmid profile and the evidence indicates that the GMRSA isolates may be related. A single plasmid of mol. wt $21.0 \times 10^6$ was responsible for resistance to penicillin in all the GMRSA isolates. This plasmid was also detected in gentamicin sensitive strains of *S. aureus* of several phage types isolated during the survey (G. Dowd, unpublished results). Chromosomally encoded penicillinase production was not detected in the GMRSA isolates although it does occur in other strains of *S. aureus* isolated in Dublin (O'Reilly *et al.*, 1981). Two different plasmids of mol. wts $24.0 \times 10^6$ and $3.0 \times 10^6$ were responsible for tetracycline resistance (Tc-R) although only 40% of the isolates carried this resistance. It is not known if the two Tc-R determinants are related or share DNA sequence homology. The $3.0 \times 10^6$ mol. wt plasmid co-migrates in agarose gels with other well characterised small Tc-R plasmids isolated in other countries.

Many of the antibiotic resistance determinants present in the isolates, including aminoglycoside, methicillin and erythromycin resistance, were not linked to detectable plasmids. Both methicillin and erythromycin resistance have been shown previously to be chromosomally encoded in some strains (Lacey, 1975). Several groups have shown that gentamicin resistance can be plasmid linked in *S. aureus* (de Saxe and Porthouse, 1979; Jaffe *et al.*, 1980; Weinstein *et al.*, 1982). Chromosomally encoded gentamicin resistance has been documented (Lacey and Lord, 1980) and may be more common than has been previously assumed. Gentamicin resistance may have been plasmid encoded in the original strains isolated in Dublin but could have been transferred subsequently to the chromosome as the number of isolates increased. The acquisition of a new antibiotic resistance determinant by *S. aureus*, especially one encoded on the chromosome, may involve a phage carrying the determinant. Transposition is likely to be involved in the integration of the resistance genes into the phage genome and the subsequent transfer of the determinant to the host chromosome. As yet we have no evidence that the GMRSA of different phage types are different strains or simply the same strain that has acquired or lost prophages.

Another approach, possibly involving biotyping, is required to differentiate between the two possibilities. The existence of antibiotic resistance determinants on the *S. aureus* chromosome may lead to stabilisation of the determinant in the hospital environment in the absence of direct antibiotic selection. This would be especially true if the determinant was located in a strain that was efficient at colonising damaged epithilial surfaces.

Because *S. aureus* plasmids are non-conjugative, bacteriophages are believed to be principally responsible for the transfer of antibiotic resistance determinants. Thus both plasmid and chromosomally encoded determinants could be transferred at similar frequencies, especially if the resistance is transposable or encoded on a large plasmid that cannot be packaged intact into phage heads.

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