Methicillin-Sensitive and -Resistant Homologues of *Staphylococcus aureus* Occur Together among Clinical Isolates

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Clinical isolates of *Staphylococcus aureus* collected from hospitals in Australia were analyzed for genetic similarities using restriction fragment length polymorphisms. Methicillin-resistant (Mc^r) isolates from Melbourne (1982) and from Hobart (1986) were closely related to a methicillin-multiresistant *S. aureus* (MRSA) strain, ANS46, originally isolated in Melbourne in 1982 and studied extensively since. Methicillin-sensitive (Mc^s) isolates were isolated concurrently with the Melbourne and Hobart Mc^r isolates. These were found to be similar to induced Mc^s variants of ANS46; these laboratory variants have lost ~40–70 kb of DNA carrying multiple resistance (R) determinants clustered around the *mec* gene. The Melbourne and Hobart Mc^s isolates appear to be natural variants lacking this region of their chromosome. A clinical Mc^r and Mc^s pair of isolates differing only in the presence of an R-cluster near the *mec* gene were also isolated in Melbourne in 1990; these are not of the same clonal line as the earlier types from Melbourne and Hobart. These data suggest that insertion or deletion (or both) similar to that produced by known mutagens occurs in the mec region of the chromosome of MRSA in clinical populations under natural selective pressures; such processes may be important in the balance of resistant and sensitive staphylococci in hospitals and other clinical environments.

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Staphylococcus aureus isolates collected in Australia during the past 10 years carry a cluster of resistance genes in the mec (methicillin resistance) region of the chromosome [1, 2]. This region also carries multiple copies of the insertion sequence IS*257* and two copies (one intact and one modified) of the transposon Tn*554* [1–4]. The mec region is unstable; Inglis et al. [5] demonstrated that the phenotypic loss of methicillin resistance under laboratory conditions of physiologic stress or in the presence of mutagenic agents such as acriflavin [5] or by repeated subculture at 37°C (unpublished observations) is due to the loss of various lengths of DNA carrying resistance genes from and around the mec region. The accumulation of resistance determinants within this region of the chromosome of methicillin multiresistant *S. aureus* (MRSA) probably has taken place over several decades of antibiotic usage [6]. These observations point to a further degree of genomic variability in *S. aureus* (at least in this part of the chromosome) beyond that provided by gene movement vectored by plasmids and phages. Thus, it would be useful to know whether variation in this region of the chromosome occurs naturally in clinical environments; such information would be valuable in establishing practices in clinical medicine that would restrain the expansion of or would deplete pools of resistance genes. We sought to answer this question by searching for methicillin-sensitive (Mc^s) homologues of MRSA strains among isolates collected from clinical environments. Such homologues have been identified in isolates from three different Australian hospitals in 1982, 1986, and 1990 and are the subject of this report.

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Materials and Methods

Bacterial isolates and strains. Three pairs of isolates collected over a period of years from hospitals in Melbourne and Hobart were examined; each pair consisted of a methicillin-resistant (Mc^r) and a Mc^s isolate (table 1). The first pair, from the Royal Melbourne Hospital in 1982, are referred to as MMc^r and MMc^s; the second pair, from Royal Hobart Hospital in 1986, are referred to as HMc^r and HMc^s; and the third pair, from the Alfred Hospital (Melbourne) in 1990, are referred to as AMc^r and AMc^s. The members of each pair were obtained from different patients within months of each other, although only in the case of the 2 Alfred strains is the sequence in which they were isolated known (table 1). MMc^r is the strain ANS46, a MRSA extensively studied in this and other laboratories [1, 5, 7–9]. Strains ANS62 and V5 are Mc^s variants of ANS46 generated in the laboratory by acriflavin treatment and aging (3-year stab culture) respectively; they have 40 and 70 kb of DNA deleted from the mec region of the chromosome [1, 4, 5].

Resistance phenotype. Antibiotic susceptibilities were tested (Sensi-Disc system; Becton Dickinson Microbiology Systems, Cockeysville, MD) using as inocula overnight cultures grown in tryptone soya broth (Oxoid, Basingstoke, UK) and diluted 1/100 in saline before swabbing onto tryptone soya agar (Oxoid) plates. After antibiotic disks were in place, plates were incu-

Table 1. Origins and resistance phenotypes of *S. aureus* strains and isolates.

Strain	Source, date [reference]	Resistance phenotype*	Resistance phenotype in mec region†
ANS46 (MMc^r)	RMH, 1982	$Km^rEm^rMc^rCm^rGm^rTc^rPc^r$	$Hg^rTc^rMc^rCd^r$
ANS62	Derived from ANS46 [1]	$Km^rEm^rMc^sCm^rGm^rTc^rPc^r$	$Hg^sTc^sMc^sCd^s$
V5	Derived from ANS46 [5]	$Km^rEm^rMc^sCm^rGm^sTc^rPc^r$	$Hg^sTc^sMc^sCd^s$
MMc^s	RMH, 1982	$Km^rEm^rMc^sCm^rGm^rTc^rPc^r$	$Hg^sTc^sMc^sCd^s$
HMc^r	RHH, 1986	$Km^sEm^rMc^rCm^rGm^rTc^rPc^r$	$Hg^rTc^rMc^rCd^r$
HMc^s	RHH, 1986	$Km^sEm^rMc^sCm^sGm^rTc^rPc^r$	$Hg^sTc^sMc^sCd^s$
AMc^r	AH, June 1990, sputum	Em^rMc^r $Gm^rTc^rPc^r$	$Hg^sTc^sMc^rCd^r$
AMc^s	AH, January 1991, burn wound	Em^rMc^s $Gm^sTc^rPc^r$	$Hg^sTc^sMc^sCd^s$

NOTE. RMH, Royal Melbourne Hospital; RHH, Royal Hobart Hospital; AH, Alfred Hospital; Km, kanamycin; Em, erythromycin; Mc, methicillin; Cm, chloramphenicol; Gm, gentamicin; Tc, tetracycline; Pc, penicillin; Cd, cadmium; Hg, mercury.

* According to Sensi-Disc method.

† Measured as described in text.

bated overnight at 37°C. Resistance to methicillin was measured the same way using disks containing 100 μg/disk and to mercuric chloride (100 μg/disk), cadmium chloride (250 μg/disk), and tetracycline (100 μg/disk). Resistance genes for these agents are clustered in the mec region of many MRSA strains [8]. Plates were incubated at 37°C for 24 h before zones of inhibition were measured. Resistance phenotypes are summarized in table 1.

DNA preparation. Preparation of genomic DNA, digestion with *Hin*dIII endonuclease, standard gel electrophoresis, and Southern blotting and probing were done as described previously [1]. Preparation of cells and digestion of their genomic DNA with *Sma*I prior to pulsed-field electrophoresis was as described previously [5]. We have shown previously that pulsed-field electrophoresis of *Sma*I digests of *S. aureus* DNA provides a sensitive method of identifying changes in the mec region of the chromosome [5].

Pulsed-field gel electrophoresis. Clamped homogeneous electric field (CHEF) gel electrophoresis was done in 1% agarose gels at 200 V for 28 h at 12–14°C (CHEF apparatus; BioRad; Richmond, CA) with 0.5× TBE (45 m*M* TRIS, 45 m*M* boric acid, and 1 m*M* EDTA, pH 8.3) with the following pulse parameters: ramped with initial forward time 1 s, final forward time 30 s. After electrophoresis, gels were stained with ethidium bromide (1 μg/mL) for 30 min, destained in water (1–6 h), and photographed over a UV transilluminator.

Southern blotting and probing of CHEF filters was done as described previously [5]. Fragments MA11, MA13, and MA14 (figure 1) from the mec region of ANS46 were used as hybridization probes.

Results

CHEF analysis after SmaI digestion of cellular DNA. CHEF analysis of *Sma*I digests of total cellular DNA showed that differences between the isolates involved only a few DNA fragments located in the 100- to 200-kb region of the gel (figure 2). The reference strain ANS46 (MMc^r) has four fragments in this region; the second of these (145 kb) carries the *mec* gene, and the fourth (110 kb) is also from the mec region [5]. In the derived Mc^s strain ANS62, the 145-kb and 110-kb fragments of ANS46 have been lost and replaced by one of 215 kb. This indicates a deletion of 40 kb of mec region DNA carrying a *Sma*I site [5]. Likewise, the clinical isolate MMc^s, isolated concurrently with but independently of ANS46 (MMc^r), has a pulsed-field fragment pattern identical to that of strain ANS62.

The HMc^r isolate is similar to the MMc^r strain except that it has an additional fragment of 185 kb. The Mc^s homologue from Hobart, HMc^s, differs from HMc^r in the same way as the laboratory-derivative V5 does from MMc^r (i.e., ANS46): Two fragments of 110 and 145 kb are lost and a new fragment of 185 kb appears, indicating deletion of ~70 kb of DNA carrying a *Sma*I site [5].

The two Alfred Hospital (1990) isolates also show a close relationship. Although the overall fragment pattern is different from that of the Melbourne (1982) and Hobart (1986) pairs, AMc^r and AMc^s have identical band patterns except that one band of AMc^r is missing in AMc^s, replaced by a fragment that is 80 kb smaller, making a single band into a doublet (figure 2). AMc^s thus appears to be the result of an 80-kb deletion from the chromosome of AMc^r. In this case the deleted DNA does not carry a *Sma*I site; the fragment pattern change is therefore different from that seen in the other two pairs of isolates.

Analysis of the mec region. To compare the chromosomal location and organization of the mec region in the different isolates, *Sma*I digests of genomic DNA were probed using cloned fragments (figure 1) from the mec region of ANS46: MA11 (4 kb carrying the *mec* gene of ANS46), MA13 (9.5 kb that carries the left end of the mec region and part of the transposon Tn*554*), and MA14 (5.8 kb that covers the right end of the mec region). MA11 hybridizes with one fragment in all Mc^r isolates (identical in size in ANS46 and HMc^r but larger in AMc^r) that is absent from all sensitive isolates and

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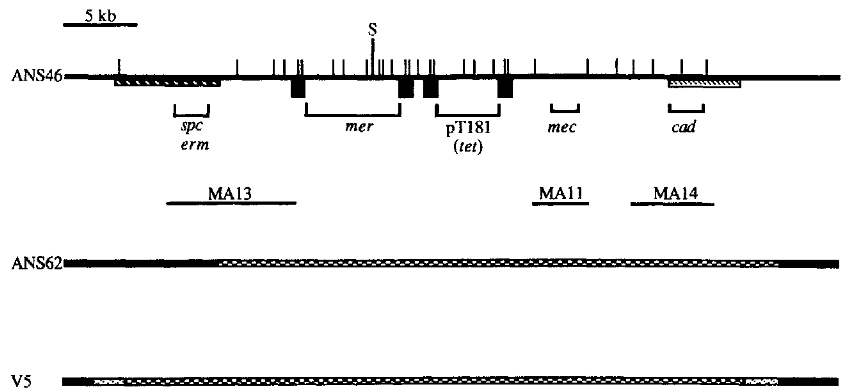

Figure 1. Map of mec region in methicillin-resistant *S. aureus* strain ANS46 (Royal Melbourne Hospital, 1982). Shaded blocks show positions of IS*257* direct repeats, hatched blocks show Tn*554* (left) and Ψ Tn*554* (right), and bracketed regions show genes for resistance to spectinomycin (*spc*), macrolides, lincosamides and streptogramin B (*erm*), mercuric ion (*mer*), tetracycline (*tet*), methicillin (*mec*), and cadmium ion (*cad*). Cloned fragments used as probes are identified below map. Endonuclease sites shown are for *Hin*dIII except for S, which is a *Sma*I site. Size bar is included. ANS62 and V5 deletion mutants are also shown: Broken lines represent DNA deleted from mec region in each; exact deletion end points of V5 are not known.

from the laboratory-deletion mutants ANS62 and V5 (figure 3).

MA13 showed a hybridization pattern identical to that of the MMc^r and the HMc^r isolates, with a strong signal detectable in a band of ~110 kb and weaker hybridization in bands of 410 and 650 kb (figure 3); the latter hybridization is

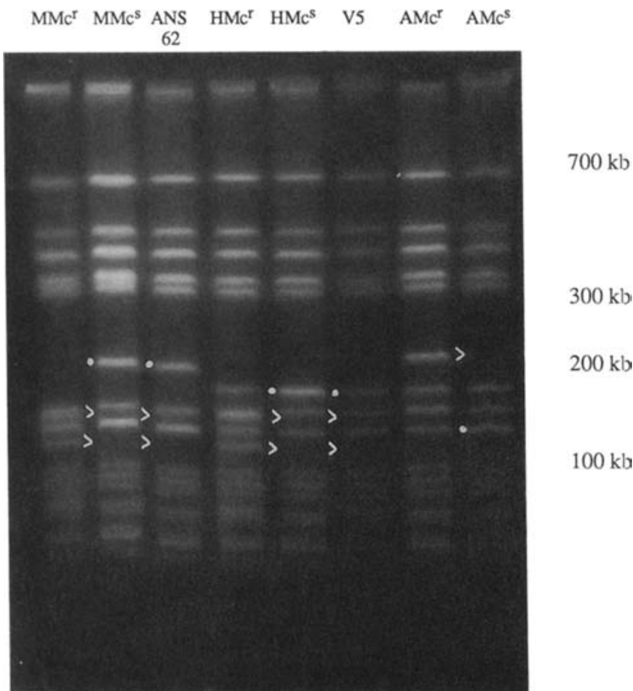

Figure 2. Clamped homogeneous electric field gel electrophoresis of *Sma*I digests of genomic DNA from *S. aureus* isolates from Melbourne (M, 1982) and Hobart (H, 1986), Alfred Hospital (A; Melbourne, 1990) methicillin-resistant (Mc^r) and -sensitive (Mc^s) pairs and Mc^s variants of ANS46. Positions of missing (>) and additional (·) fragments are indicated. Doublet bands are identified by comparison with neighboring bands of same strain (e.g., AMc^s). Size scale is at right. Some variation in migration rate is seen across gel.

due to the presence of additional copies of Tn*554* located remote from the mec region in the chromosome [4]. These weakly hybridizing fragments are seen in all isolates. The MMc^s isolate is identical to strain ANS62, characterized by an additional strongly hybridizing fragment at 215 kb. The HMc^s isolate was found to have a hybridization pattern identical to that of V5 (figure 3), where only the remote copies of Tn*554* hybridize to MA13, indicating the absence from V5 and HMc^s of further DNA homologous with MA13 (the deletion in V5 is greater than in ANS62, spanning 70 kb and extending leftward beyond Tn*554*, figure 1). Neither AMc^r nor AMc^s carries DNA homologous with MA13 in or near the mec region. MA14 showed hybridization patterns identical to those obtained with MA11 (data not shown).

For more detailed mapping of similarities and differences within the mec region, DNA cut with *Hin*dIII (generating a large number of fragments from the *S. aureus* chromosome) was probed with MA11, MA13, and MA14. As with the *Sma*I hybridization patterns, MA11 showed no homology to fragments from the sensitive isolates but hybridized strongly to a fragment of identical size in all resistant isolates (figure 4A).

MA13 hybridized to five *Hin*dIII bands of ANS46 (figure 4B); an identical pattern was seen with the HMc^r isolate. The MMc^s isolate showed a hybridization pattern identical to that of ANS62, where the three fragments corresponding to the right side of MA13 (2.7, 0.9 and 0.7 kb bands) are missing, whereas that on the left side (8 kb) is present. The 7-kb band hybridizing to the MA13 segment is a result of hybridization with the two remote copies of Tn*554* referred to earlier [4]. Again, the HMc^s isolate showed a pattern identical to that of strain V5 (figure 4B), where only the additional copies of Tn*554* hybridize to MA13, indicating the absence of Tn*554* from the mec region as discussed earlier. Likewise, both AMc^r and AMc^s isolates have hybridization patterns indicating that only Tn*554* copies remote from the mec region are present (figure 4B).

MA14 hybridized to three *Hin*dIII fragments in ANS46

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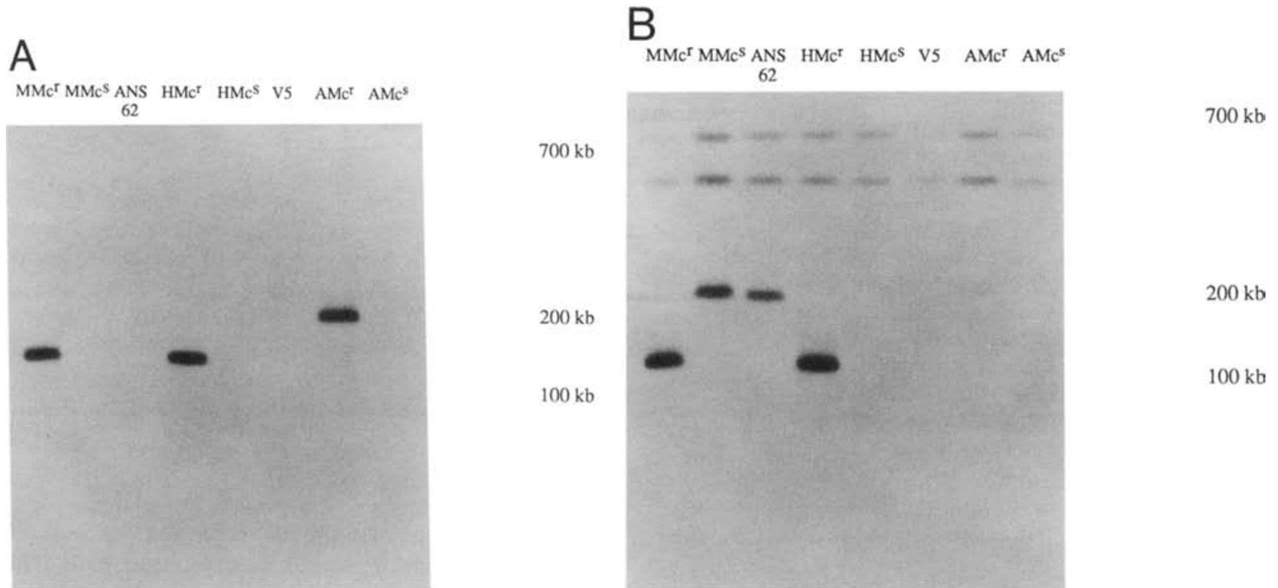

Figure 3. Southern transfer of *Sma*-cut DNA from *S. aureus* (figure 2) to nitrocellulose filter showing (**A**) hybridization with MA11 (only methicillin-resistant [Mc^r] isolates hybridize with MA11) and (**B**) hybridization with MA13 (melbourne [M] and Hobart [H] Mc^r isolates show an identical fragment hybridizing strongly with MA13 at ~110 kb); faint hybridization seen in both isolates at ~145 kb is due to hybridization with IS*257*, as MA13 carries ~80 bp of IS*257*). MMc^s (sensitive) isolate shows a pattern identical to that of ANS62 where MA13 hybridizes with newly generated fragment at ~215 kb. Weak hybridization to MA13 is identical in all isolates (differences seen in strength of signal are due to differences in loading of DNA, figure 2). Faint hybridization to 215-kb fragment of Alfred Hospital Mc^r (AMc^r) is due to incomplete stripping of filter.

(figure 4C); an identical pattern was seen across all the resistant isolates, but no homology was detected in the sensitive isolates. The DNA absent from the sensitive members of the 1982, 1986, and 1990 clinical pairs thus includes that lost from the acriflavin-induced or physiologically stressed strains (ANS62 and V5).

Discussion

The skin and upper respiratory tract represent the major natural habitat of *S. aureus*, with 20%–50% of the human population carrying this microorganism. The development of multiple resistance to antimicrobials in staphylococci has been the subject of a great deal of speculation, but there seems to be little doubt that in present-day clinical populations of these bacteria particular clones or strains serve as reservoirs of resistance genes that can be transferred to newly evolving clones or strains. The effects of sustained abstinence of antibiotic use could be important in eliminating such pools of resistance genes. Our own experience is that isolates of *S. aureus* taken from the community at large (i.e., away from hospitals) carry few resistance genes and are highly variable genetically, as measured by chromosomal restriction fragment length polymorphism analysis (RFLP); clinical MRSA isolates, by contrast, particularly those taken during outbreaks, are largely clonal and carry apparently unselected resistance genes [10].

The genetic instability of the mec region of the chromosome points to the likelihood that the cluster of resistance genes located here will slowly disappear from MRSA populations in the absence of antibiotic use. While it would be satisfying to believe that such deletional processes underlie what is observed in the Mc^s clinical isolates examined here, the sample size is small, and in any case we cannot be certain whether resistant strains precede sensitive ones or vice versa.

Our analyses have involved two approaches using RFLP analysis. The first, represented by the *Sma*I digestion profiles (figure 2), indicates similarities between isolates at the level of the total genome. The *Sma*I RFLP data show that the HMc^r isolate is closely related to strain ANS46 (isolated 4 years earlier), differing by only one additional genomic fragment in HMc^r. Similarities in RFLPs are a measure of similarities in DNA sequence throughout the genome of the isolates [10–12]. These two resistant strains belong to a clonal type we refer to as type 46 (identical or closely related to the MMc^r strain ANS46), which appears to have been dominant in clinical infections during the 1980s (unpublished data). The MMc^s and HMc^s isolates show the loss of DNA sequences apparently identical to those of the induced and mapped Mc^s mutants ANS62 and V5, respectively, and thus are simple deletion homologues of the resistant types MMc^r and HMc^r. AMc^r is not a type 46 isolate (unpublished data). However, it shares identical DNA sequences to the right boundary of the mec region of type 46, sequences that are

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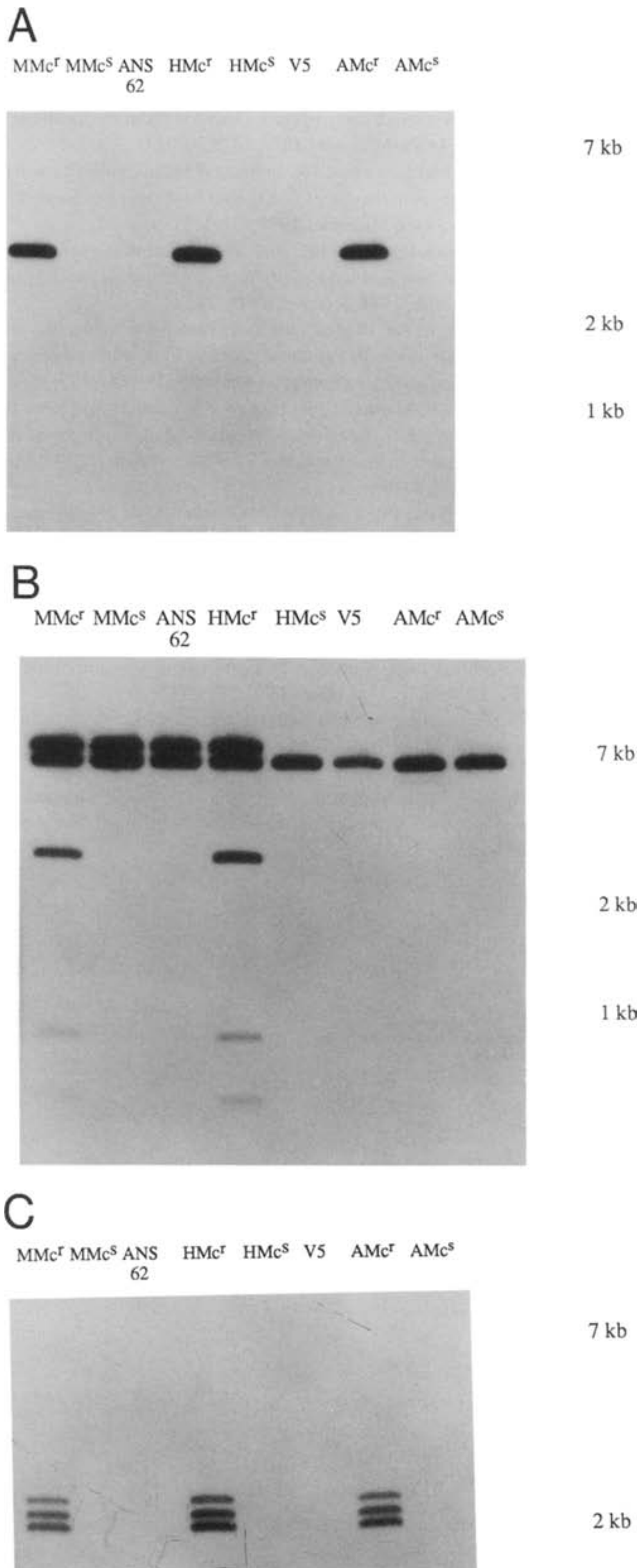

Figure 4. Southern transfer of *Hin*dIII-cut DNA from *S. aureus* (isolates as in figure 2) to nitrocellulose filter showing **A**, hybridization with MA11, which hybridizes to fragment of identical size (4 kb) in all methicillin-resistant (Mc^r) isolates; **B**, hybridization with MA13, which shows identical pattern in both Melbourne (M) and Hobart (H) Mc^r isolates (MA13 hybridizes with same five bands in each). MMc^s (sensitive) is identical to ANS62. HMc^s is identical to V5. Signal detected at 6.9 kb is due to hybridization with remote copies of Tn*554;* and **C**, hybridization with MA14, where identical hybridization pattern is detected in Mc^r isolates, but no homology is seen with Mc^s isolates.

lost from the AMc^s strain as in the induced type 46 Mc^s mutants ANS62 and V5. Thus, the AMc^s strain is likewise a deletion homologue of the resistant type AMc^r.

In the second approach we examined *Hin*dIII hybridization patterns to obtain a detailed analysis of the extent of similarities between the isolates within that part of the chromosome carrying the *mec* gene and associated resistance determinants. The *mec* gene was found to be carried on a *Hin*dIII fragment that was the same size in the chromosome of all 3 resistant isolates tested, covering 1982–1990. Probing with cloned mec region fragments from ANS46 confirmed the absence of certain segments of this region in the clinical sensitive types, identical to segment deletions that have been experimentally generated from ANS46 [5]. The MMc^s isolate appears to be missing ~40 kb of DNA and lacks the resistance determinants *cad*A, *mec*A, *tet*A, and *mer*A *mer*B; its phenotype is thus identical to that of ANS62 (table 1).

It is important to note that the loss of *tet*A from the mec cluster did not generate a tetracycline-sensitive mutant but resulted in a reduced level of resistance. This is explained by the fact that ANS46 (hence ANS62 and V5) carries an additional *tet* gene elsewhere in the chromosome [5]. This appears also to be the case for the Melbourne and Hobart isolates examined here. However, the Alfred Hospital isolates did not show this effect, and we conclude therefore that there is no tetA locus within the mec region of the Alfred Hospital isolates. The HMc^s isolate shows the absence of a longer section of the mec region, as is seen in the V5 deletant, in which the deletion extends leftward to remove the Tn*554* copy to the left of *mer*A *mer*B [5]. The Alfred Hospital pair (AMc^r and AMc^s, 1990) is significantly different from ANS46 (unpublished data) but is closely related to the recently described Canberra type II group [10]. The AMc^r isolate carries a *Hin*dIII fragment containing *mec*, identical to that in ANS46, which is absent from the AMc^s isolate, suggesting some similarities in the organization of the mec region in the 1990 Mc^r isolate and the 1982 isolate even though they differ more generally.

Thus, strains of Mc^s *S. aureus* can be isolated from hospital patients that correspond to those that can be generated from a Mc^r MRSA strain in the laboratory.

Whether the clinical Mc^s isolates represent progenitors of MRSA awaiting the capture and insertion of appropriate clusters of R-genes or are (like the laboratory strains) the result of lost chromosomal resistance gene clusters, we can-

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not tell. The difference, however, may be an important one in determining strategies of MRSA control in clinical practice. The fact that the 1990 AMc^s types were isolated some months after the AMc^r types were detected (table 1) is suggestive but by no means conclusive that deletion has resulted in the loss of resistance genes. The question now to be addressed is whether limitations on the administration of antibiotics and antimicrobials would result in the loss of *mec* and associated chromosomal R-clusters from existing MRSA types and thus generate sensitive types. A clear loss of R-genes from the mec region of multiple isolates correlating with constrained antibiotic usage would indicate that deletional loss is the basis of the present observations.

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