Amplification of a Section of Chromosomal DNA in Methicillin-resistant
Staphylococcus aureus following Growth in High Concentrations
of Methicillin

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Growth of two independently isolated strains of methicillin-resistant Staphylococcus aureus (MRSA) in increasing concentrations of methicillin (step-selection) resulted in increased resistance in these strains. When chromosomal DNA from the step-selected variants was probed using DNA sequences previously demonstrated to be associated with methicillin resistance in MRSA strains, amplification of the homologous chromosomal sequence was identified. Growth of these step-selected strains in the absence of methicillin resulted in loss of the amplified sequence, while the original sequence remained. There are differences between the two strains in the stability of maintenance of amplified sections. Prolonged storage of the variants on a high concentration of methicillin resulted in loss of amplified sections without concomitant loss of methicillin resistance. Thus amplification may be only one of at least two molecular mechanisms available to S. aureus to increase methicillin resistance in response to step-selection. Probing of cells of the highly resistant sub-population of a heterogeneously resistant MRSA strain showed that duplication of this mec-associated DNA is not involved in the mechanism of heteroresistance.

INTRODUCTION

The biochemical and genetic basis of methicillin resistance in Staphylococcus aureus has been studied for more than 20 years. Although the biochemical mechanism underlying methicillin resistance has been attributed to the production of a new penicillin-binding protein (PBP2' or 2a) with decreased affinity for methicillin and other β-lactams (Fontana, 1985; Hartman & Tomasz, 1986; Matsuhashi et al., 1986; Reynolds & Fuller, 1986), the nature of the genetic determinant for this protein, and the regulation of its expression, have yet to be described. The role of PBP2a in relation to unusual features of the resistance observed in most, but not all, clinical strains, is also unclear. These features include resistance heterogeneity (sub-clonal variation of resistance) and susceptibility to environmental conditions of the resistance (for review see Matthews & Stewart, 1984), features which occur to varying degrees in different strains of methicillin-resistant S. aureus (MRSA). Thus high salt concentration in the growth medium has been reported to enhance the resistance of some strains (Dyke, 1969; Sabath et al., 1972) but to decrease that of others (Heneine & Stewart, 1986), while the effect of lower incubation temperatures is to enhance, but to varying degrees, the resistance of most strains studied. Heterogeneity is also variable between different strains, and among heterogeneously resistant (heteroresistant) strains the stability of resistance in the most resistant minority sub-population varies characteristically from strain to strain (Hartman & Tomasz, 1986).

One explanation of resistance heterogeneity may be that variable or intermittent gene amplification occurs in different clones within a population. As a first approach to this proposal,

Abbreviations: MRSA, methicillin-resistant Staphylococcus aureus; PBP, penicillin-binding protein.

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we have sought to determine whether resistant sub-clones in a population, selected by challenge with appropriate increments of methicillin concentration, carry the same or increased amounts of sequences associated with methicillin resistance. To do this we probed total DNA from three MRSA strains and their highly resistant variants with mec-associated DNA cloned from one of the strains (Matthews et al., 1987).

Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype/genotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANS46</td>
<td>Mc^K Km^K Tc^K Hg^K Cd^R Nv^S Cm^R Er^R Tm^R Sm^R</td>
<td>Clinical isolate from K. Harvey, Royal Melbourne Hospital, Victoria, Australia, 1982 [also described as RM4 (Heneine &amp; Stewart, 1986)]</td>
</tr>
<tr>
<td>ANS47</td>
<td>Mc^K Km^K Tc^K Hg^K Cd^R Nv^S Cm^S Er^R Tm^R Sm^R</td>
<td>Clinical isolate from K. Harvey, Royal Melbourne Hospital, Victoria, Australia, 1982 [also described as RM5 (Heneine &amp; Stewart, 1986)]</td>
</tr>
<tr>
<td>C5</td>
<td>Mc^K Km^K Tc^K Hg^K Cd^R Nv^S Tm^K</td>
<td>From S. Cohen, Michael Reese Hospital, Chicago, USA. Originally isolated in Seattle, USA, 1967</td>
</tr>
<tr>
<td>ANS46(1000)</td>
<td>As for ANS46 with Mc^K step-selected to higher level</td>
<td>Matthews et al. (1987)</td>
</tr>
<tr>
<td>C5(1000)</td>
<td>As for C5 with Mc^K step-selected to higher level; Hg^K Cd^S due to loss of pIC5</td>
<td></td>
</tr>
<tr>
<td>ANS46(1000st)</td>
<td>As for ANS46(1000), stored for 18 months as described in Methods</td>
<td></td>
</tr>
<tr>
<td>C5(1000st)</td>
<td>As for C5(1000), stored for 18 months as described in Methods</td>
<td>This study</td>
</tr>
<tr>
<td>ANS46(3000)</td>
<td>As for ANS46(1000st) with Mc^K step-selected to higher level</td>
<td></td>
</tr>
<tr>
<td>C5(3000)</td>
<td>As for C5(1000st) with Mc^K step-selected to higher level</td>
<td></td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>pMF5</td>
<td>Bg\text{II} fragment MF5 (3-5 kb) from ANS46 chromosome cloned into the Bg\text{II} site of pUC9 (Matthews et al., 1987)</td>
<td></td>
</tr>
</tbody>
</table>

METHODS

Bacterial strains and plasmids. The clinical MRSA strains used were ANS46, ANS47 (strains RM4 and RM5, respectively, of Heneine & Stewart, 1986) and C5 (Cohen & Sweeney, 1970); these strains are of Australian (ANS46 and 47) and American (C5) origin. Resistance characteristics of these strains and their variants are given in Table 1. The step-selection of ANS46 and C5 to generate the variants ANS46(1000) and C5(1000) (capable of growth in shaken cultures at 1000 pg methicillin ml^-1) has been described (Matthews et al., 1987). The variants ANS46(3000) and C5(3000) were step-selected in the same way from stocks of ANS46(1000) and C5(1000) which had been stored on LG agar (Matthews et al., 1987) containing 1000 μg methicillin ml^-1 at 4 °C, with two-monthly sub-culturing, for 18 months [these stored stocks are denoted ANS46(1000st) and C5(1000st)]. Strain C5 contains the plasmid pIC5, which was lost in the step-selection of C5(1000). The plasmid pMF5 is a recombinant of the vector pUC9 and MF5, a 3-5 kb Bg\text{II} fragment of ANS46 chromosomal mec-associated DNA (Table 1). Its mec-associated DNA insert is identical to that isolated from a methicillin resistant transductant by Beck et al. (1986) and Matthews et al. (1987).

Population analysis of cultures for methicillin resistance. Strains were grown as 20 ml shaken cultures with or without the addition of methicillin. The survival of sub-populations of resistant cells at various methicillin concentrations (efficiency of plating – e.o.p.) was determined as described previously (Heneine & Stewart, 1986).

DNA methods. S. aureus total DNA was prepared as described previously (Matthews et al., 1987). For strain variants grown in different concentrations of methicillin, the nomenclature used to designate a particular culture or DNA preparation is written x(y)z, with x the strain name, y the limit concentration of methicillin (in μg ml^-1)
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Fig. 1. Population analysis of strains. The e.o.p. of the strains and variants, grown with and without methicillin, was determined as described by Heneine & Stewart (1986). Cultures tested were: ANS47(0)0 (○), ANS47(0)50 (●), ANS46(0)0 (□), C5(0)(△), and ANS46(0)50, ANS46(1000)100, ANS46(1000)0, ANS46(1000st)1000, ANS46(1000st)0, C5(0)50, C5(1000)1000, C5(1000)0, C5(1000st)1000 and C5(1000st)0 (■).

to which the variant was step-selected, and x the concentration of methicillin in which the variant was grown for DNA preparation.

Plasmid DNA was prepared from Escherichia coli by the method of Maniatis et al. (1982). Restriction endonucleases were purchased from Pharmacia and used according to the supplier’s instructions. Agarose gel electrophoresis was by standard methods (Maniatis et al., 1982) in Tris/acetic acid buffer, using agarose concentrations of 0.35–1.0% (w/v) and voltage gradients of 0.5–2.0 V cm⁻¹, depending on the molecular mass of the DNA fragments being separated. Transfer to nitrocellulose membranes was by the method of Southern (1975), and hybridization of [³²P]DNA to nitrocellulose membranes was by the method of Johnson et al. (1984), at 42°C with the addition of 50% (v/v) formamide to the hybridization solution. Nick-translation of plasmid DNA (0.5 µg per hybridization) was as described previously (Stewart et al., 1985). To quantify certain hybridization analyses, selected bands and a background sample of equal size were excised from the nitrocellulose membrane and the radioactivity was assayed in a Beckman liquid scintillation counter to 2% error (Young, 1984). Acid depurination and fragmentation of DNA was done to ensure uniform transfer of DNA fragments of different molecular mass from gels to filters (Wahl et al., 1979). Fragmentation of probe DNA was achieved in the same way to ensure uniform hybridization of probe to homologous molecules on filters.

RESULTS

Population analysis of strains

Strain ANS47, and strains ANS46, C5 and the variants step-selected to 1000 µg methicillin ml⁻¹, were grown overnight at 37°C in shaken broth cultures (containing appropriate concentrations of methicillin), and the e.o.p. on increasing concentrations of methicillin was determined (Fig. 1). ANS46, C5 and their variants display a homogeneous distribution of resistance at all but high drug concentrations, with growth in methicillin and step-selection of resistance having a relatively small effect on the resistance of the majority population. For strain ANS47, however, the resistance distribution is typical of heteroresistant MRSA, with growth in moderate concentrations of methicillin increasing the resistance of the majority of cells by 10–100-fold and the proportion of resistant cells by 1000–10000-fold. This strain also shows enhanced resistance at 30°C (Heneine & Stewart, 1986), and is thus described as thermosensitive heterogeneous MRSA, as defined by Hartman & Tomasz (1986).

Detection and mapping of a chromosomal amplification in ANS46(1000)

The cloning and mapping of 24 kb of ANS46 chromosomal DNA associated with methicillin and other resistances was described by Matthews et al. (1987); the restriction endonuclease map
of this DNA segment, with additions arising from the present study, is shown in Fig. 2. The DNA preparations ANS46(0)0 and ANS46(1000)1000 were digested with the restriction endonucleases HaeIII, HpaII, BglII, PstI, HindIII, XbaI, EcoRI, XhoI, BamHI, EcoRI/XhoI and BamHI/XhoI. All digests were probed with labelled pMF5 DNA; Fig. 3(a) shows the results for the HaeIII, HpaII, BglII, PstI and HindIII digests. The hybridization observed with ANS46(0)0 digests enables partial extension of the restriction endonuclease map (Fig. 2) to include the first HindIII, HaeIII, XbaI, EcoRI and XhoI sites to the right of the cloned 24 kb region. These were determined from the size of strongly hybridizing fragments unaccountable by the previously cloned and mapped fragments, and therefore overlapping the right side of MF5. Certain of the faintly hybridizing fragments result from hybridization of MF5 to the IS257-like repeat of approximately 1 kb (Matthews et al., 1987; Gillespie et al., 1987), which is present in MF5 and at three other locations in the 24 kb of cloned DNA. When these repeats are taken into account, the intensity of hybridization of pMF5 to fragments mapping within or overlapping the right side of MF5 in the mapped DNA approximately doubles in ANS46(1000)1000 compared with ANS46(0)0, while hybridization with the fragment overlapping the left side of MF5 remains at the same intensity, and a new fragment hybridizes with an intensity comparable with this. We deduce that a new section of DNA with substantial homology to MF5 has been created during the step-selection of ANS46( low), and that this has most likely occurred by the duplication of MF5 plus at least 15 kb of DNA to its right, including the rightwards EcoRI and XhoI sites shown in Fig. 2.

The sizes of the new fragments created by this duplication, or amplification as it will now be referred to, enable the mapping of the first endonuclease site immediately to the left of the 'insertion' site of the amplified section (Fig. 2). As no new BglII fragments were observed in ANS46(1000)1000, the left end of the amplified section must lie in the 0-17 kb between the left side of the new copy of MF5 (a BglII fragment) and the new HindIII site 0-17 kb to its left. Whether the amplified section occurs in tandem with the original sequence, or is inserted remotely in the chromosome, cannot be deduced from these data. Nor can any precise estimate of its length be made, although the location of XhoI sites approximately 15 kb to the right of MF5, and approximately 30 kb to the left of the new copy of MF5, indicates that the amplified section must be at least 48 kb long if it is present as a tandem repeat.
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Detection and mapping of a chromosomal amplification in C5(1000)

The DNA preparations C5(0)0 and C5(1000)1000 were also digested with the restriction endonucleases HaeIII, HpaII, BglII, PstI, HindIII, XbaI, EcoRI, XhoI, BamHI, EcoRI/XhoI and BamHI/XhoI. All digests were probed with labelled pMF5 DNA; Fig. 3(b) shows the results for the HaeIII, HpaII, BglII, PstI and HindIII digests.

Faintly hybridizing fragments, which occur in C5(0)0 but not in C5(1000)1000 DNA, are due to the presence in strain C5 of the plasmid pIC5, which specifies production of penicillinase and mercury resistance. This plasmid carries two copies of the IS257-like repeat, but was lost during the step-selection of C5(1000) (Matthews et al., 1987). When these bands are taken into account, the same general changes appear to have accompanied the C5 to C5(1000) transition as in the ANS46 to ANS46(1000) transition, namely, that fragments corresponding in size to those mapping within or overlapping the right side of MF5 in ANS46 approximately double in hybridization intensity, another fragment remains unchanged, and a new fragment is created. Results not fitting this pattern for HaeIII digestion (Fig. 3b, lanes 1 and 2) are due to two pIC5 HaeIII fragments co-migrating, or one of these co-migrating with the 3.8 kb chromosomal fragment. In the HpaII digest the new fragment found is slightly larger than the original fragment.

Although the sequences on the C5 chromosome that hybridize with MF5 have not been cloned and mapped, it appears that the same event has occurred in the step-selection of C5 to C5(1000) as for that of ANS46 to ANS46(1000), namely, the amplification of a section of DNA that includes a sequence with identity or close homology to MF5. Assuming this, it is then possible to construct restriction endonuclease maps of the first sites to the left of the fragments in C5 corresponding to MF5 for HindIII, PstI, HpaII, HaeIII, XbaI, EcoRI, XhoI and BamHI, both in the original (C5) and amplified positions [C5(1000)] (Fig. 2). Again, the creation of no...
Table 2. Copy ratios of amplified sections in step-selected variants

Values for radioactivity are c.p.m. for the excised bands, after subtraction of radioactivity of an appropriate excised background sample.

<table>
<thead>
<tr>
<th>Variant DNA</th>
<th>Radioactivity of amplified band</th>
<th>Radioactivity of original band</th>
<th>Copy ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANS46(1000)1000</td>
<td>2173</td>
<td>2321</td>
<td>0.94</td>
</tr>
<tr>
<td>ANS46(1000)0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ANS46(1000st)1000</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ANS46(1000st)0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ANS46(3000)3000</td>
<td>63</td>
<td>1858</td>
<td>0.14†</td>
</tr>
<tr>
<td>ANS46(3000)0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C5(1000)1000</td>
<td>3896</td>
<td>3849</td>
<td>1.0</td>
</tr>
<tr>
<td>C5(1000)0</td>
<td>456</td>
<td>3200</td>
<td>0.14</td>
</tr>
<tr>
<td>C5(1000st)1000</td>
<td>905</td>
<td>3806</td>
<td>0.24</td>
</tr>
<tr>
<td>C5(1000st)0</td>
<td>175</td>
<td>3457</td>
<td>0.05</td>
</tr>
<tr>
<td>C5(3000)3000</td>
<td>7086</td>
<td>2142</td>
<td>3.3</td>
</tr>
<tr>
<td>C5(3000)0</td>
<td>4062</td>
<td>1679</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* Copy ratio determined by dividing first value by second.
† Copy ratio for this variant determined as described.

ew MF5-hybridizing BglII fragment, and the presence this time of a HaeIII site 0.7 kb from the left side of the MF5 fragment in the amplified position, indicate that the left-hand end of the amplified section is located within this 0.7 kb. However, the map of the amplified segment in C5 can be seen to diverge significantly from that found in ANS46, with no endonuclease sites conserved rightwards from a point approximately 3 kb to the right of the MF5 fragment.

Stability of the amplification

To assess the effect of growth with and without methicillin, the variants ANS46(1000) and C5(1000) were grown both in the presence and absence of methicillin, and total cell DNA was prepared and probed to estimate the copy number of the amplified section compared to that of the original section; this proportion is termed the amplification copy ratio. To do this, DNA from ANS46 variants was digested with HpaII, and that from C5 variants with PstI. After Southern transfer and hybridization of the digests with 32P-labelled pMF5 DNA the copy ratio of the amplified section was determined by excising bands and counting the bound radioactivity of relevant hybridizing fragments, then subtracting that of appropriate background samples. The hybridization of the left section of MF5 with the original homologous fragment, represented by this corrected radioactivity, was divided into that of the same section of MF5 with the new, amplified homologous fragment. As the length of membrane-bound DNA homologous to the probe is the same in both cases, the ratio of the bound radioactivity of the relevant bands gives a direct measurement of amplification copy ratio (Table 2). For the ANS46/HpaII digests, the original fragment is 3.0 kb long and the new, amplified fragment 2.6 kb (Fig. 3a). For C5/PstI digests, the two fragment sizes are 6.2 and 14 kb respectively (Fig. 3b).

As can be seen from Fig. 4(a), growth of the variant ANS46(1000) without methicillin resulted in complete loss of at least that part of the amplified section homologous to pMF5 DNA, whereas the variant C5(1000) suffered only a partial loss of this part of the amplified region, with the copy ratio falling from 1.0 to 0.14. This apparent difference in the stability of the region between the two strains was examined in the variants ANS46(1000st) and C5(1000st), which were generated by storing ANS46(1000) and C5(1000) on LG agar containing methicillin for 18 months as described in Methods. These were grown both with and without 1000 μg methicillin ml⁻¹ [to give the DNA preparations x(1000st)1000 and x(1000st)0, where x is the strain designation] and the DNA was digested and probed as before (Fig. 4b). The differing stability of the amplified section is again reflected in these results, with the DNA preparations ANS46(1000st)1000 and ANS46(1000st)0 both showing complete loss of the amplified region homologous to MF5 DNA, C5(1000st)1000 showing amplification at a copy ratio of 0.24 (Table 2) and C5(1000st)0 showing incomplete loss of the region (copy ratio 0.05).
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Fig. 4. Stability of the amplified segments. Chromosomal DNA from the x(1000) (a), x(1000st) (b) and x(3000) (c) variants, grown with (odd-numbered lanes) and without (even-numbered lanes) the relevant concentration of methicillin, was digested with HpaII (ANS46 variants; lanes 1 and 2) or PstI (C5 variants; lanes 3 and 4). (d) ANS46(3000)3000 DNA digested with BglII (lane 1), HaeIII (lane 2) and HindIII (lane 3), with bands appearing on step-selection indicated by arrows. All digests were electrophoresed in 1-0% agarose gels, transferred to nitrocellulose and hybridized to 32P-labelled pMF5 as described. Molecular size markers correspond to digests of λ DNA with HindIII and pUC9 DNA with HindIII/DraI. The gels in (a) were run at a higher voltage gradient than the others, and therefore require a separate marker lane. The right hand marker lane applies to (b), (c) and (d).

The two variants ANS46(1000st) and C5(1000st) were subjected to further step-selection, undergoing a rapid increase in resistance to 3000 µg methicillin ml⁻¹ over five sub-culturings with 1 in 100 inocula (i.e. 30–40 generations). They were then grown in 3000 µg methicillin ml⁻¹ and without methicillin to prepare the DNAs x(3000)3000 and x(3000)0, and appropriate endonuclease digestions of these DNAs probed as before to examine any amplification (Fig. 4c). The DNA preparation ANS46(3000)0 showed no evidence of amplification, but ANS46(3000)3000, while showing no amplification band at 2-6 kb in a HpaII digest, did show a faint new band at 2-2 kb. Further probing of BglII, HaeIII and HindIII digests of this DNA preparation revealed no new BglII bands, but single faint, new HaeIII and HindIII bands of 1-6 and 3-8 kb respectively (Fig. 4b). The HpaII digests (Fig. 4c, lanes 1 and 2) also show bands at approximately 5 and 6 kb of considerably less intensity than the new band at 2-2 kb. These may represent another structural rearrangement occurring at a low frequency, but are considered more likely to result from partial digestion of the DNA or perhaps low frequency point mutations at restriction sites.

When excised and counted the HaeIII band at 1-6 kb showed approximately 0-034 of the radioactivity of the band at 8-6 kb, indicating a copy ratio of this probable new amplification of approximately 0-1. This tripling of the ratio from 0-034 to 0-1 allows for the presence of three copies of the IS257-like repeat on the 8-6 kb HaeIII fragment, and depends on the fact that hybridization involves only the repeat part of MF5, as the HaeIII site in MF5 occurs just to the right of the repeat (Fig. 2). The new 2-2 kb HpaII fragments also indicates that the left-hand end of this new amplified section lies within 0-3 kb of the left end of MF5, as the left HpaII site in MF5 occurs 1-9 kb to the right of this point.
The DNAs C5(3000)3000 and C5(3000)0 both showed the presence of the original amplification with strong PstI bands at 14 kb (Fig. 4c, lanes and 4), which when excised and counted indicated further amplification to copy ratios of 3.3 and 2.4 respectively. It is interesting to note that in neither of these DNAs were any new bands seen, apart from the one at 14 kb. This observation of the presence of no additional bands [apart from that already seen in C5(1000)1000] applied to all digests of these DNAs, i.e. PstI, EcoRI, XhoI, EcoRI/XhoI, BamHI and BamHI/XhoI (data not shown).

Southern hybridization analysis of DNA from ANS47

To test for the possible involvement of the amplification described above in the mechanism of heterogeneity of methicillin resistance, ANS46, C5 and the heteroresistant strain ANS47 were grown in the presence and absence of 50 μg methicillin ml⁻¹; the cell populations were analysed for methicillin resistance (Fig. 1), and total cell DNA was prepared from the remaining cultures. The DNA preparations ANS47(0)0 and ANS47(0)50 were digested with HpaII, PstI, EcoRI, XhoI and EcoRI/XhoI, and probed with ³²P-labelled pMF5. This revealed identical hybridization patterns to those of ANS46(0)0, with no change apparent following growth in 50 μg methicillin ml⁻¹. Growth of ANS46 and C5 in the same concentration of methicillin, while showing a relatively small increase in the proportion of cells resistant to high concentrations of methicillin (Fig. 1), also showed no such evidence of amplification (data not shown).

Further probing (with pMF5) of ANS47(0)0 DNA digested with HindIII, HaeIII and XbaI was also done to search for differences in the hybridization pattern between this DNA and that from ANS46. No differences in band number, mobility or hybridization intensity were detected (data not shown), thereby implying that there is no difference between ANS46 and ANS47 in arrangement of the three resistance determinants and the four copies of the IS257-like repeat (Matthews et al., 1987), contained in the approximately 30 kb of chromosomal sequences covered by this analysis.

DISCUSSION

The gene amplification events described in this report, occurring as they do in two different MRSA strains (of distinctly different geographical and temporal origins) in response to the same stimulus of step-selection by increasing concentrations of methicillin, have implications in understanding the genetic basis of methicillin resistance in S. aureus. Amplification of chromosomal resistance determinants in response to growth in elevated drug concentrations, and subsequent decrease in determinant copy ratio on growth in drug-free medium, has been observed in a number of organisms and with a number of drugs (Stark & Wahl, 1984; Wilson & Morgan, 1985; Young, 1984). Such copy ratio instability was also shown for the amplification of the mec-associated sequence studied here, as the variants of both strains showed a rapid loss of the amplified sequence on overnight culture without methicillin.

The observation that this instability was more pronounced in ANS46 than in C5, and that the initial step-selection occurred more readily in ANS46 (Matthews et al., 1987), could be explained by lower stability of the ANS46 chromosome in this region, an interesting possibility in view of the presence of four copies of the IS257-like repeat in the region (Fig. 2), compared with only one in C5. Indeed, the proximity of the left end of the amplified section to the left side of the IS257-like repeat largely contained in MF5, in all three amplifications mapped, is further circumstantial evidence for involvement of IS257 in site-specific recombination, adding to the weight of such evidence implicating this ubiquitous element in transposition of staphylococcal resistance genes (Gillespie et al., 1987; Lyon & Skurray, 1987).

The probable arrangement and location of the amplified sections in the two strains, that is, whether amplification occurred in tandem or remote from the original section, remains a matter for conjecture. No evidence can be adduced for either possibility for the amplifications observed in ANS46 variants. However, in the variants of C5 the further amplification of the duplicate section beyond a copy ratio of one [in C5(3000)3000], without the creation of any additional
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MF5-hybridizing fragments, would seem to favor tandem duplication. For a remotely located duplicate section to be further amplified without creating any additional fragments (in the same way that new fragments were created by the first duplication) would require amplification (in tandem or otherwise) of that section plus at least 26 kb to its left, in order to encompass all those sites to its left mapped from the first set of new fragments (Fig. 2) and so create no additional fragments. If the original duplication [in C5(1000)1000] occurred in tandem these sites would represent sites at the right end of the duplicated section, and the new fragments would simply increase in copy number and hybridization intensity (as observed) on additional tandem amplification.

Step-selection of C5(1000st) to grow on 3000 µg methicillin ml⁻¹ was accompanied by a substantial amplification (copy ratio approximately 3-3), whereas the corresponding step-selection of ANS46(1000st) resulted in a much lower degree of amplification (copy ratio approximately 0-1). This may indicate that complete loss of the original amplification [in ANS46(1000)] occurred during the prolonged storage, so that not even a small proportion (<1%) of [ANS46(1000st)] cells carrying it remained to be selected by the increasing methicillin concentrations. The occurrence of an apparently different amplification in ANS46(3000) to that observed in ANS46(1000) raises questions as to the specificity of the amplification events seen in this strain, and further experiments are planned to investigate this and the configuration of the ANS46 duplications.

The smaller increase in copy ratio of the new amplification (from zero to 0-1), measured during the step-selection of ANS46(1000st) to ANS46(3000), compared to that for the C5(1000st) copies in the same step-selection (from 0-25 to 3-3), suggests that if amplification is indeed a cause of increased methicillin resistance, it may be less important in explaining the increased methicillin resistance of the ANS46(3000) variant than in the C5(3000) variant. This observation is consistent with the different stabilities of the amplifications in the two strains during extended storage on a high concentration of methicillin. The fact that both stored variants retained their ability to grow in 1000 µg methicillin ml⁻¹ despite the complete loss of amplified MF5 sequences in ANS46(1000st), and their partial loss in C5(1000st), suggests either that MF5 does not carry sequences directly specifying methicillin resistance, and is lost separately from other amplified sequences, or that another mechanism (other than gene amplification) exists for elevating methicillin resistance. The former seems unlikely in view of the endonuclease site similarly between MF5 and the gene encoding PBP 2a (following paper: Inglis et al., 1988). The latter possibility, an alternative mechanism, could be based on enhanced expression (e.g. induction) of the gene(s) in the amplified segment, or involve a separate pathway for resistance. It may involve the same mechanism whereby sensitive laboratory strains can be step-selected to moderate and high levels of methicillin resistance (Heneine & Stewart, 1986) in the absence of these mec-associated sequences in the cells (P. R. Matthews & N. Heneine, unpublished observations).

The possible involvement of the amplification phenomenon described here in the mechanism of heterogeneity, whereby only a small fraction of the cell population displays resistance (Hartman & Tomasz, 1986), was tested by looking for the occurrence of such an amplification on selection of the minority resistant population of a heteroresistant strain. We chose the thermosensitive heteroresistant strain ANS47 as being representative of MRSA strains which show these characteristics, and selected the resistant population of cells by growth in a moderate concentration of methicillin (50 µg ml⁻¹). Probing of DNA from these resistant cells demonstrated that no amplification of the mec-associated fragment MF5 occurs, thus discounting the likelihood that the mechanism of heterogeneity involves amplification of this section of chromosomal DNA. These results do not, of course, exclude the possibility that the mechanism of heterogeneity involves modulation of the expression of this mec-associated sequence, either by change of the sequences to the right of MF5 or by some other mechanism.

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