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**REVERSION OF A METHICILLIN RESISTANT
STAPHYLOCOCCUS AUREUS STRAIN TO
SENSITIVITY *IN VIVO***

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ABSTRACT

In 1986 during an outbreak of MRSA infection at Palmerston North Hospital an MRSA strain (PN MRSA) was recovered from a patient who was treated and subsequently discharged. In 1990 prior to readmission an isolate of *S.aureus* which produced small colonies typical of MRSA was recovered from the same patient. This isolate was resistant to several antibiotics but unexpectedly was sensitive to methicillin. This investigation examines the possibility that this atypical methicillin sensitive *S.aureus* (AMSSA) strain was derived *in vivo* from the resistant strain, possibly by a reversible mutation, and examines the possibility that exposure of this sensitive strain to analogues of methicillin may lead to reversion to resistance.

The PN MRSA and the AMSSA strain were compared with various other methicillin resistant and sensitive staphylococci by phage typing, reverse phage typing, plasmid profiles, and total genomic digests using the restriction enzymes *HindIII* and *SmaI*. In all instances results showed that the PN MRSA and the AMSSA strain were more similar to each other than they were to any of the other staphylococci examined.

Probing of total genomic and *SmaI*-digested DNA with the methicillin resistance gene *mec* showed that the gene was present in all the 'high level multiply resistant' and 'low level singularly resistant' MRSA strains examined but absent from the AMSSA strain and the other methicillin sensitive isolates. The 143 kb fragment which contained the *mec* gene in the PN MRSA was absent from the *SmaI* restriction profile of the AMSSA strain. The loss of this fragment and another fragment (104 kb) followed by the gain of a 203 kb fragment in the profile of the AMSSA strain was consistent with a deletion (44 kilobases) which spans a *SmaI* site. The deletion corresponds to the estimated size of the *mec* gene complex.

Overall the results suggest that the AMSSA strain was derived *in vivo* from the PN MRSA strain and in the process 44 kilobases of DNA was deleted from the *mec* region. As sensitivity in the AMSSA strain was not due to an easily reversible point mutation or small deletion it is unlikely that the isolate will rapidly develop resistance to methicillin following exposure to the drug. However the results suggest that the *mec* region is unstable and that under the appropriate conditions the *mec* region may be lost from the chromosome of MRSA strain *in vivo*.

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CHAPTER 1

INTRODUCTION TO THESIS

Methicillin resistant strains of *Staphylococcus aureus* (MRSA) are important nosocomial pathogens as they are usually resistant not only to methicillin but also to a wide range of unrelated antibiotics such as erythromycin, chloramphenicol, tetracycline, and aminoglycosides (Thornsberry 1988). This makes MRSA infections difficult to treat. The only antibiotic to which all strains of MRSA are susceptible is vancomycin (Hackbarth and Chambers 1989b) but this is expensive, may produce side effects, and if used frequently could lead to the development of vancomycin resistant strains.

Methicillin resistance in *S.aureus* is due to the production of a novel penicillin binding protein, PBP2a, which has a low affinity for methicillin (Chambers 1988). PBP2a is encoded by the *mec* gene which resides on the chromosome. The region of DNA surrounding the *mec* gene harbours many other resistance determinants, the number, nature and arrangement of which can vary significantly among different isolates (Inglis *et al* 1990, Dubin *et al* 1992). This region can be up to 50 kilobases in size and has no allelic equivalent in the chromosomes of methicillin sensitive strains of *S.aureus*.

The elucidation of the *mec* gene complex and further studies to investigate the genetic mechanisms leading to the acquisition of resistance determinants in the *mec* region have largely involved the conversion, *in vitro*, of methicillin resistant strains to sensitivity and the subsequent study (in the MRSA strain) of that segment of DNA lost in the sensitive derivative (Inglis *et al* 1990). The MRSA strains were converted *in vitro* to sensitivity using techniques such as acriflavin treatment (Matthews *et al* 1987), curing of penicillinase plasmids followed by growth in antibiotic free medium (Wada *et al* 1991), growth at elevated temperatures, exposure to ultraviolet radiation, and starvation (Inglis *et al* 1990). The size of the deletion in these strains varied considerably but could be up to 250 kilobases in size. In most cases one or both of

the deletion endpoints occurred at or near either a transposon attachment site or the insertion sequence IS257 (Inglis *et al* 1990, Wada *et al* 1991).

Spontaneous *in vivo* loss of resistance to methicillin has not to our knowledge been reported. Although recent evidence by Inglis *et al* (1993) suggests that deletion of large amounts of DNA may occur in the *mec* region of the chromosome of MRSA in the clinical environment. However in that study the methicillin resistant and sensitive isolates examined were from different patients and the sequence in which they were recovered was not known in two of the three cases. Therefore the differences seen may have been due to insertion rather than deletion, of DNA. This thesis reports evidence for the loss of methicillin resistance *in vivo*.

In 1986, during an outbreak of MRSA infection at Palmerston North hospital, an MRSA strain (PN MRSA) was recovered from a patient who was treated and subsequently discharged. In 1990 prior to readmission an isolate of *S.aureus*, which produced small colonies typical of an MRSA strain, was recovered from the same patient. This isolate was resistant to several antibiotics but unexpectedly was sensitive to methicillin. The medical microbiologist at Palmerston North hospital was concerned that this atypical methicillin sensitive *S.aureus* (AMSSA) strain (which was assumed to be derived from the PN MRSA strain) could rapidly become resistant to methicillin if the patient was treated with that antibiotic.

This thesis reports an investigation into this possibility and addresses the question as to whether minor, easily reversible, changes (eg point mutations) led to sensitivity or, in contrast to the above, sensitivity is due to the loss of the *mec* gene. Consequently this investigation addresses four related questions:

- 1) Are the properties of the original (MRSA) isolate and the second (methicillin sensitive) isolate so similar as to imply that one was derived from the other?.
- 2) Was sensitivity in the AMSSA strain due to a reversible mutation such as a point mutation or a small deletion? or
- 3) Does the sensitive strain lack the entire *mec* gene? And if so,
- 4) How much DNA has been lost by the sensitive strain?

HISTORICAL REVIEW

1.1 HISTORICAL PERSPECTIVE

Before the antibiotic era, the prognosis for patients with severe *Staphylococcus aureus* infection was often very poor. The introduction of penicillin into clinical use in the early 1940's brought about a dramatic reversal in this situation (Lyon and Skurray 1987, Thornsberry 1988). For the first time, invasive *S.aureus* infections, such as those that develop from accidental or operative trauma, burns, and other serious skin lesions, could be treated effectively. However, this was to be short lived. Within a few years Kirby (1944) reported the isolation of an enzyme from a strain of *S.aureus* that could inactivate penicillin. This enzyme called penicillinase or β -lactamase, catalysed the hydrolysis of the β -lactam ring of penicillin, forming penicillonic acid, which had no antibactericidal properties (Thornsberry 1988). The gene which encoded resistance to penicillin was borne on a plasmid (Thornsberry 1988) and so spread rapidly. By 1946 it was estimated that 60% of hospital isolates in the United Kingdom were resistant to penicillin (Barber and Rozwadowska-Dowzenko 1948, Lyon and Skurray 1987). By the 1950's the high prevalence of staphylococci resistant to penicillin had seriously reduced the value of this antibiotic and alternative agents were needed.

The introduction of methicillin, the first of the penicillinase-resistant semisynthetic penicillins, into clinical practice in 1959 and 1960 solved this problem for a time (Chambers 1988, Thornsberry 1988). Strains of *S.aureus* resistant to methicillin were detected almost immediately (Jevons 1961). However, these strains accounted for only approximately 1% of isolates from the United Kingdom (Lyon and Skurray 1987) and were not considered to pose a serious threat to the overall effectiveness of the antibiotic.

MRSA strains were isolated with increasing frequency in Europe and Asia throughout the 1960's (Lyon and Skurray 1987, Cookson and Phillips 1990), however there was a general decline in their prevalence internationally in the early 1970's (Casewell 1986). In the late 1970's MRSA reemerged causing larger and more widespread

outbreaks and, in contrast to the strains isolated earlier, a higher proportion of these new strains were resistant to several antibiotics (Lyon and Skurray 1987, Thornsberry 1988). These multiply resistant MRSA strains have continued to cause major problems in hospitals worldwide to the present day.

1.2 MRSA IN NEW ZEALAND

Methicillin resistant strains of *S.aureus* (MRSA) were first isolated in New Zealand in 1975 (Humble 1976). From 1975 to 1984 relatively few cases were identified with a maximum of 13 infections in any one year (CDNZ suppl.1 1992). In 1985 however, the number of cases began to increase and from 1985 to 1987 two major outbreaks in Palmerston North and Wellington occurred, involving 258 persons at Palmerston North Hospital and 79 persons at Wellington Hospital (Jones 1987, Scrimgeour 1987, Martin 1987, Martin *et al* 1989). Although fewer cases have been reported in subsequent years, MRSA continues to affect a substantial number of persons in New Zealand (Heffernan *et al* 1993).

In contrast to MRSA overseas, the majority of MRSA in New Zealand in recent years are not resistant to multiple antibiotics (CDNZ suppl.1 1992, Heffernan *et al* 1993). Furthermore, the majority of MRSA isolates display only low level methicillin resistance with minimum inhibitory concentrations (MICs) to methicillin of 8 to 32 µg/ml (CDNZ suppl.1 1992, Heffernan *et al* 1993).

1.3 HETEROGENEOUS AND HOMOGENEOUS RESISTANCE

Heterogeneous Resistance

Most strains of methicillin resistant staphylococci are heterogeneous in their expression of resistance (Chambers 1988). Within a population of a methicillin resistant strain the majority of cells show only a low level of resistance, whereas minor subpopulations demonstrate resistance to higher concentrations of methicillin (Chambers 1988, Hackbarth and Chambers 1989). The proportion of cells that

express higher resistance levels is reproducible but strain dependent, and ranges from 10^{-2} to 10^{-8} (Tomasz *et al* 1991, Berger-Bachi *et al* 1992).

Expression of resistance is enhanced by growth in high concentrations of methicillin because the susceptible subpopulation is eliminated and the highly resistant subpopulation is selected. These antibiotic-selected cells are more uniformly resistant than the parent strain, however, this trait is unstable, and so with repeated subculturing in drug-free medium the culture reverts to its heterogeneous pattern of resistance (Tomasz *et al* 1991, Hackbarth and Chambers 1989b).

Homogeneous Resistance

A minority of strains are homogeneous in their expression of resistance ie cells are uniform in their expression of resistance and can grow in high concentrations of methicillin (Chambers 1988, Tomasz *et al* 1991). They maintain this trait even with repeated subculturing in the absence of antibiotics.

1.4 MECHANISM OF METHICILLIN RESISTANCE

PBPs

β -lactam antibiotics (such as penicillin and methicillin) act by inhibiting enzymes involved in assembling the bacterial cell wall (Lyon and Skurray 1987, Chambers 1988, Hackbarth and Chambers 1989a). These enzymes are found in the membrane and catalyze the cross-linking reactions between peptidoglycan polymers. The cross-linking gives the wall additional rigidity, which is essential to maintain the viability of the cell. β -lactam antibiotics covalently bind to the active site of these enzymes and inhibit cross-linking (Waxman and Strominger 1983, Hackbarth and Chambers 1989). This leads to weakening of the cell wall and ultimately lysis. Because these enzymes bind penicillin at their active site they are called penicillin binding proteins (PBPs). The fundamental difference between susceptible staphylococci and methicillin resistant strains is their PBPs.

Methicillin susceptible strains of *S.aureus* produce four or five PBPs. PBPs 1, 2, 3, 3', and 4 with approximate molecular weights of 85, 80, 75, 70, and 45 kilodaltons

respectively (Wyke *et al* 1984, Chambers 1988). The specific physiologic function or functions of these staphylococcal PBPs as transpeptidases, endopeptidases, and carboxypeptidases (the three enzymatic activities which may be possessed by PBPs) have not been defined completely. However PBPs 1, 2, and 3 appear to be necessary for cell growth and survival (Georgopapadakou *et al* 1986, Reynolds 1988).

PBP2a

Methicillin resistant strains of *S.aureus* produce an additional 78 kilodalton PBP, termed PBP2a which has a low binding affinity for β -lactam antibiotics (Chambers 1988, Hackbarth and Chambers 1989a). Methicillin resistant strains of coagulase negative staphylococci also produce PBP2a (Chambers 1987).

In contrast to other staphylococcal PBPs, which generally bind β -lactam antibiotics at low concentrations, PBP2a binds β -lactam antibiotics only at a relatively high antibiotic concentration (Brown and Reynolds 1980, Chambers 1988). Since the presence of PBP2a confers methicillin resistance presumably it can substitute for the 'normal' PBPs when these have been saturated by drug, ie PBP2a can perform the functions necessary for cell wall assembly (Chambers 1988).

1.5 GENETICS OF METHICILLIN RESISTANCE

Mec

The genetic determinant which confers methicillin resistance is termed *mec* (Chambers 1988, Berger-Bachi 1989, Hackbarth and Chambers 1989a). Initial confusion over whether *mec* resided on plasmid or chromosomal DNA has been resolved. Evidence for a plasmid location was indirect and based on characteristics of elimination of *mec* from resistant strains (Chambers 1988). However, some conditions associated with the elimination, transduction, and transformation of *mec* suggested that the determinant was chromosomal. Transformation of *mec* by chromosomal but not plasmid DNA (Sjostrom *et al* 1975) and elucidation of its map location to the *pur-nov-his* gene cluster (Kuhl *et al* 1978) have conclusively demonstrated that the determinant is chromosomal.

Stewart and Roseblum (1980) have shown using cotransduction studies, that no allele equivalent to *mec* exists in susceptible strains of *S.aureus*. Beck and colleagues (1986) confirmed this by showing that *mec* probes hybridised with chromosomal DNA from unrelated resistant strains, but not with chromosomal DNA from susceptible strains.

***Mec* encodes PBP2a**

The PBP2a gene has been shown to be part of *mec* (Hackbarth and Chambers 1989a). Transformation of *mec* from *S.epidermidis* to a susceptible strain *S.carnosus* caused the recipient strain to produce PBP2a (Tesch *et al* 1988), and DNA from the transformants hybridised with *mec* specific DNA (Beck *et al* 1986), whereas DNA from the recipient strain did not. Furthermore, a 4 kilobase fragment of *mec* has the same restriction enzyme map as a cloned fragment which produces PBP2a (Matsuhashi *et al* 1986, Inglis *et al* 1988).

Origin of *mec*

Southern blot analysis suggests a unique origin of *mec* and indicates that the gene is highly conserved (Beck *et al* 1986, Song *et al* 1987, Hackbarth and Chambers 1989a). Likewise, the PBP2a gene product is also highly conserved structurally. After partial proteolytic digestion PBP2a's from several unrelated strains of *S.aureus* and coagulase negative staphylococci had identical fragments which were different from those of other staphylococcal PBPs (Hackbarth and Chambers 1989a).

The PBP2a gene has been cloned into *E.coli* and sequencing data suggests that there is homology with PBPs from other organisms. (Matsuhashi *et al* 1986, Song *et al* 1988). The regulatory region and the first 300 base pairs of the gene are similar to those of a staphylococcal penicillinase gene. Thus PBP2a may have evolved from the fusion of the staphylococcal penicillinase gene and a PBP from a nonstaphylococcal source. Song and coworkers (1987) noted similarities at the amino acid level, of *mec* to the genes encoding PBP2 and PBP3 of *E.coli*. However the degree of similarity of these PBPs is not consistent with a close evolutionary relationship which might be observed in a recent horizontal transfer event from *E.coli* (Wu *et al* 1992). The overall G+C content of the *mec* gene is approximately 30.5% (Wu *et al* 1992)

(Staphylococcal special average 30 to 35% G+C (Novick 1990)). This observation is in opposition to the roughly 50% G+C content observed in *E.coli* DNA (Wu *et al* 1992). Furthermore, the high degree of identity at the DNA level (>99%) among *mec* genes from distant geographical origins suggests that relatively little time has passed since the *mec* gene entered staphylococci (Wu *et al* 1992). Thus it appears likely that the *mec* gene was transferred to staphylococci in a relatively recent horizontal transfer event from a species which perhaps shares the low G+C content characteristic of staphylococcal DNA. Candidate donor organisms which might fill the role include members of the genera *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Lactobacillus* or *Sarcina* (Wu *et al* 1992).

Regulation of PBP2a production

PBP2a is inducible by β -lactam antibiotics although the protein can be produced constitutively (Chambers 1988, Hackbarth and Chambers 1989a). PBP2a is inducible in strains in which the penicillinase plasmid is present and constitutive in strains lacking the plasmid (Ubukata *et al* 1985). Thus the repressor of the penicillinase gene may play a role in the regulation of PBP2a.

A repressor may also be contained within *mec* (Hackbarth and Chambers 1989a). An upstream open reading frame is present on the strand of DNA opposite that which encodes PBP2a (Song *et al* 1987). An open reading frame of similar location is associated with penicillinase genes in other bacterial species, and its product is believed to be a repressor (Hackbarth and Chambers 1989a). This may explain why in some strains PBP2a is inducible even in the absence of the penicillinase plasmid.

Transposition of *mec*

The insertion sequence IS257 (or IS431) has been found to be closely associated with the *mec* gene in all methicillin resistant strains of *S.aureus* studied so far (Barberis-Maino *et al* 1987, Matthews *et al* 1987, Wada *et al* 1991, Dubin *et al* 1991, Dubin *et al* 1992). Homologous (IS257-like) sequences have been shown to flank genes encoding resistance to mercury, tetracycline, kanamycin, and trimethoprim found either on plasmids (such as the penicillinase plasmid), or on the chromosome of methicillin resistant strains of *S.aureus* (Skinner *et al* 1988, Hackbarth and Chambers

1989, Dubin *et al* 1991). IS257 has also been found on the chromosome of methicillin sensitive strains of *S.aureus* (Barbaris-Maino *et al* 1987).

Evidence suggests that IS257 may play a role in the transfer of *mec* between strains and the subsequent integration of *mec* into the chromosome (Hackbarth and Chambers 1989). When *mec* was transformed from *S.epidermidis* into a susceptible strain of *S.carnosus* (Tesch *et al* 1988), the cloned fragment that conferred resistance did not integrate into the chromosome but remained on the plasmid (Tesch *et al* 1988). Not surprisingly no IS257-like sequences were detected on the *S.carnosus* chromosome. In addition *mec* cannot be transduced into a methicillin sensitive strain of *S.epidermidis* that is sensitive to mercury or cadmium (Blanchard *et al* 1986), presumably because the proper insertion sequences required for integration are missing.

Trees and Iandolo (1988) demonstrated that the penicillinase plasmid (which contains IS257) has an active role in the transduction of methicillin resistance into susceptible strains by providing a temporary insertion sequence for the *mec*-containing transposon. This requirement of penicillinase for the transduction of *mec* into *S.aureus* has been known for some time (Stewart and Rosenblum 1980). Perhaps *mec* insertion into the penicillinase plasmid is necessary for it to acquire the information needed to integrate into its chromosomal site.

Genetic Basis for the Accumulation of resistance determinants in the *mec* region

MRSA strains are usually resistant not only to methicillin but also to a wide range of unrelated antibiotics (Lyon and Skurray 1987) and in such multiply resistant strains the DNA surrounding the *mec* gene often contains these resistant determinants (Inglis *et al* 1990, Dubin *et al* 1992). This region varies significantly among different isolates and may be up to 50 kilobases in size (Inglis *et al* 1990, Dubin *et al* 1991, Dubin *et al* 1992). No allele equivalent to *mec* or its surrounding DNA has been found in the chromosome of methicillin sensitive strains of *S.aureus* (Hackbarth and Chambers 1989).

Physical mapping of the *mec* region of the chromosome of various isolates (Inglis *et al* 1990, Dubin *et al* 1991, Wada *et al* 1991, Dubin *et al* 1992.) has shown that these resistant determinants are all part of transposable elements. Resistance genes are

either:

- 1) flanked by direct repeats of the insertion sequence IS257 eg the mercury resistance operon (Inglis *et al* 1990),
- 2) part of a transposon, eg the macrolide-lincosamide-streptogramin B (MLS) resistance genes in Tn554, or the cadmium resistance gene in UTn554 (Inglis *et al* 1990, Chikramane *et al* 1991 Dubin *et al* 1992),or
- 3) part of plasmids which have integrated into the chromosome and are flanked by direct repeats of IS257, eg the tetracycline resistance plasmid, pT181 and the kanamycin resistance plasmid pUB110 (Dubin *et al* 1991)

The genetic basis for the accumulation of these resistant determinants in the *mec* region of the staphylococcal chromosome could be explained by either or both of the following:

- 1) Firstly, the region may have been created by the insertion of a plasmid which had previously assembled these resistance genes and insertion sequences (Inglis *et al* 1990). Large staphylococcal plasmids carrying multiple resistance genes and insertion sequences such as IS257 have been described (Lyon and Skurray 1987, Skurray *et al* 1988).
- 2) Secondly, features of this region of the chromosome might have promoted recombination by providing target sites for homologous recombination or for transposon insertion (eg Tn554 only integrates at specific chromosomal attachment sites (Chikramane 1991)). Insertion sequences (eg IS257) or transposon attachment sites might function as target sequences in sensitive strains, which could provide the recombinational sites for resistance genes coming into the cell on plasmids, phages, or conjugative transposons (Inglis *et al* 1990).

Other Factors

The mechanisms and genes which govern the switch from low level resistance to various higher resistance levels in heterogeneous strains of MRSA is as yet undetermined. The level of resistance perhaps surprisingly does not correlate with the quantity of PBP2a present (Chambers 1988, Murakami and Tomasz 1989). It is known that additional chromosomally located genes that are not linked to *mec* are essential for the expression of methicillin resistance (Berger-Bachi 1983, Berger-

Bachi and Kohler 1983, Kornblum *et al* 1986). These genes termed *fem* factors (factors essential for the expression of methicillin resistance) are found in both the chromosome of methicillin resistant and sensitive strains (Berger-Bachi 1992). Two of these factors, *femA* and *femB* are involved in the peptidoglycan cross-bridge formation of *S.aureus* peptidoglycan (Berger-Bachi 1992, Henze *et al* 1993). Inactivation of these genes by Tn551 insertion lowers the level of methicillin resistance (Berger-Bachi 1989).

1.6 SUMMARY

Methicillin resistance is genetically and biochemically complex. PBP2a, the protein associated with methicillin resistance is encoded by the *mec* gene, a fusion product between a staphylococcal penicillinase gene and a PBP from a nonstaphylococcal source. Two different repressors may control expression of methicillin resistance. However within a single population various subpopulations may express different levels of resistance. What governs the switch from low level resistance to various higher levels is unknown. However the level of resistance does not correlate with the with the quantity of PBP2a present. Other factors not linked to *mec* are known to influence the level of methicillin resistance.

MRSA strains are usually resistant not only to methicillin but also to a wide range of unrelated antibiotics, and in such multiply resistant strains, the region of DNA surrounding the *mec* gene harbours many of these resistance determinants. This region can be up to 50 kilobases in size and has no allelic equivalent in the chromosomes of methicillin sensitive strains of *S.aureus*.

Clearly methicillin resistance is a system of unusual complexity which merits further investigation.