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 *Hin*dIII and *Sma*I. 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 *Sma*I-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 *Sma*I 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 consistant with a deletion (44 kilobases) which spans a *Sma*I 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 IS*257* (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 ß-lactamase, catalysed the hydrolysis of the ß-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, Hacbarth 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

ß-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 ridigity, which is essential to maintain the viability of the cell. ß-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 ß-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 ß-lactam antibiotics at low concentrations, PBP2a binds ß-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 horizonal 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 ß-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 IS*257* (or IS*431*) 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 (IS*257*-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). IS*257* has also been found on the chromosome of methicillin sensitive strains of *S.aureus* (Barbaris-Maino *et al* 1987).

Evidence suggests that IS*257* 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 IS*257*-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 IS*257*) 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 IS*257* eg the mercury resistance operon (Inglis *et al* 1990),

2) part of a transposon, eg the macrolide-lincosamide-streptogramin B (MLS) resistance genes in Tn*554*, or the cadmium resistance gene in UTn*554* (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 IS*257*, 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 IS*257* 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 Tn*554* only integrates at specific chromosomal attachment sites (Chikramane 1991)). Insertion sequences (eg IS*257*) 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, *fem*A and *fem*B are involved in the peptidoglycan cross-bridge formation of *S.aureus* peptidoglycan (Berger-Bachi 1992, Henze *et al* 1993). Inactivation of these genes by Tn*551* 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.

CHAPTER 2
COMPARISON OF THE COLONY MORPHOLOGY, GROWTH RATE, AND MINIMUM INHIBITORY CONCENTRATION OF METHICILLIN FOR VARIOUS *STAPHYLOCOCCUS AUREUS* STRAINS

2.1 INTRODUCTION

The minimum inhibitory concentration (MIC) of methicillin for various *S.aureus* isolates was defined, as were their colony morphology and growth rate to see if there is a correlation between antibiotic sensitivity, colony morphology and growth rate.

2.2 MATERIALS

S.aureus Isolation

DNase Test Agar	
DNA	2 g
Tryptose (Bacto)	20 g
NaCl	5 g
Agar	15 g
Water to	1000 ml

Agar Dilution Method

12800 μg/ml Methicillin. Stock Solution	
Methicillin	0.128 g
Distilled water to	10.0 ml

Preparation of the Standard Inoculum	
0.048 M $BaCl_2$	
$BaCl_2.H_20$	1.173 g
Sterile distilled water to	100.0 ml

0.36 M H_2SO_4 (1% v/v)	
Sterile Brain Heart Infusion broth	99.0 ml
concentrated H_2SO_4	1.0 ml

0.5 McFarland Standard	
0.048 M $BaCl_2$	0.5 ml
0.36 M H_2SO_4	99.5 ml

Place 5.0 ml aliquots into sterile tubes, seal tightly and store in the dark.
Equivalent to a bacterial culture with a density of 10^8 organisms/ml

0.15 M Saline (Physiological salt concentration)	
NaCl	8.78 g
Distilled water to	100.0 ml

Autoclave at 121°c for 15 minutes and store at room temperature.

Table 2.1.
***S.aureus* isolates studied in this thesis**

Description of isolates	Source of isolates
Palmerston North outbreak MRSA (PN MRSA)	Patient x: Palmerston North Hospital
Atypical methicillin sensitive strain (AMSSA)	Patient x: Palmerston North Hospital
Wellington outbreak MRSA (WN MRSA)	Wellington Hospital
MRSAT	Palmerston North Hospital
MRSAB	Palmerston North Hospital
MRSAG	Palmerston North Hospital
Methicillin sensitive isolate (SA1)	Palmerston North Hospital
S.aureus isolates (SA2, SA4, SA201, SA203, SA209, SA214, SA217, SA301, SA303, SA304, SA312)	Nasal swab from healthy individuals
MRSA (ST90 410)	Auckland Hospital
MRSA (ST90 371)	Auckland Hospital
MRSA (ST90 236)	Rotorua Hospital
MRSA (ST90 211)	Auckland Hospital
MRSA (ST90 176)	Auckland Hospital
MRSA (ST90 103)	Auckland Hospital
MRSA (ST88 1377)	Auckland Hospital
MRSA (ST90 267)	Palmerston North Hospital
MRSA (AS89 103)	Dunedin Hospital
MRSA (AS89 105)	Wellington Hospital
MRSA (ST90 380)	Palmerston North Hospital
MRSA (ST89 580)	Auckland Hospital (Singapore isolate)
MRSA (ST89 1302)	Auckland Hospital (Japan isolate)
MRSA (ST88 988)	Auckland Hospital (Fiji isolate)

2.3 METHODS

2.3.1 *S.AUREUS* STRAINS

All the strains which were studied in this thesis are listed in Table 2.1.
The PN MRSA, the AMSSA strain, three methicillin resistant strains MRSAT, MRSAB, MRSAG, and the methicillin sensitive isolate SA1 were provided by Dr Gordon Scrimgeour of the Palmerston North Hospital.
The WN MRSA and all the other methicillin resistant strains (ST# and AS#) were kindly provided by Helen Heffernan of the New Zealand Communicable Disease Centre.
All the other *S.aureus* isolates (SA#) were recovered from nasal swabs of healthy individuals on the assumption that 30% of healthy adults are colonised with *S.aureus*. (Davis *et al* 1990).

2.3.2 ISOLATION OF *S.AUREUS* FROM HEALTHY ADULTS

A swab wetted in 0.15 M saline was rotated around the inside of the nostril. The swab was spread over the surface of a BHI agar plate and the plate incubated at 37°c overnight. White or golden colonies, 1 to 3 mm in size, were tested for catalase activity. Catalase positive organisms were Gram stained and tested for DNase and coagulase activity.

1) Catalase Test

A section of a colony was picked with a toothpick and added to a drop of hydrogen peroxide on a glass slide. The production of bubbles indicates catalase activity.

2) Gram stain

A small amount of culture was added to a drop of water on a glass slide. The drop was allowed to air dry and was then heat fixed. The slide was stained with crystal violet for 60 seconds, then Gram's iodine for 60 seconds, destained with 95% Alcohol for 1-2 seconds, and counterstained with Safranin/Carbol fushin for 60

seconds. The slide was then examined under the microscope at 1000x magnification. Staphylococcal species appear as Gram positive (purple) cocci in grape-like clusters.

3) DNase Test

A colony was picked with a straight wire and stabbed into a DNA plate. The plate was incubated at 37°c overnight. The plate was then flooded with 1 M HCL and left at room temperature for 15 minutes. A clearing around the inoculum indicates DNase activity.

4) Coagulase Test

A single colony was added to a tube containing 0.5 ml of rabbit plasma and incubated at 37°c. The mixture was examined for clotting at 30 minute intervals over 4 hours.

Isolates were identified as *Staphylococcus aureus* if they were catalase positive, DNase positive, coagulase positive, and produced Gram positive cocci in clumps.

2.3.3 SCREENING OF *S.AUREUS* STRAINS FOR METHICILLIN RESISTANCE

AGAR DILUTION METHOD

A stock solution of methicillin (12800 µg/ml) was diluted from 1280 µg/ml to 20 µg/ml using the scheme outlined in Table 2.2 (pg 18)(recommended by NCCLS 1990). 2 ml of each antibiotic solution was then added to 38 ml of Mueller Hinton agar at 50°c, mixed, and poured into two petri dishes on a level surface. 2 ml of sterile distilled water was added to 38 ml of agar for control plates.

Preparation of the Standard Inoculum

The standard inoculum was prepared by touching the top of four or five colonies and inoculating them into a universal bottle containing 5.0 ml of Brain Heart Infusion (BHI) broth. This suspension was incubated at 30°c on a rotary shaker until it reached a turbidity equivalent to that of a 0.5 McFarland standard (=10^8 colony

forming units per ml). The suspension was then diluted 10-fold by adding 1 ml of bacteria to 9 ml of sterile 0.15 M saline.

Inoculation of Plates

1 µl of the diluted bacterial suspension was spotted onto a Mueller Hinton agar plate using a calibrated loop to give a final inoculum of approximately 10^4 CFU on an area 5 to 8 mm in diameter. 32 strains were spotted onto each plate using a grid. A plate containing no methicillin was inoculated first. Then starting at the lowest concentration, the plates containing methicillin were inoculated. Finally another control plate (no methicillin) was inoculated to ensure that there was no contamination or antimicrobial carry over during inoculation. Once the inoculum spots were dry the plates were incubated at 30°c for 16 to 20 hours.

The MIC of a strain was taken as the lowest concentration of antibiotic that inhibited growth. A few small colonies or a flat haze were disregarded.

2.3.4 COLONY MORPHOLOGY

A 100 µl aliquot of *S.aureus* cultures stored at -70° were thawed and inoculated into 5 ml of BHI broth. The cultures were incubated at 37°c overnight on a rotary shaker. A loopful was streaked to produce isolated colonies on a BHI plate. Plates were incubated at 37°c for 24 hours and photographed.

2.3.5 GROWTH RATES

A loopful of culture, obtained from growth on a fresh BHI plate, was inoculated into 100 ml of BHI broth and incubated at 37°c on a rotary shaker. The absorbance at 600 nm was recorded at 30 minute intervals.

Table 2.2.
Scheme for Preparing Dilutions of Methicillin to be used in Agar Dilution Susceptibility Tests (NCCLS 1990)

Step	Antimicrobial Solution			MHB[a] Vol =	Final MHB[a] Conc	Final MHA[b] Conc
	Conc	Source	Vol +			
1	12800 ug/ml	stock	1 ml	9 ml	1280 ug/ml	64 ug/ml
2	1280	step 1	1 ml	1 ml	640	32
3	1280	step 1	1 ml	3 ml	320	16
4	1280	step 1	1 ml	7 ml	160	8
5	160	step 4	1 ml	1 ml	80	4
6	160	step 4	1 ml	3 ml	40	2
7	160	step 4	1 ml	7 ml	20	1

a. MHB = Mueller Hinton Broth
b. MHA = Mueller Hinton Agar

2.4 RESULTS

2.4.1 MINIMUM INHIBITORY CONCENTRATION

The minimum concentrations of methicillin which inhibited the growth of various *S.aureus* strains are shown in Table 2.3 (pg 21).

An isolate is considered resistant (NCCLS 1990) if its minimum inhibitory concentration (MIC) is ≥8 μg/ml. The degree of antibiotic resistance shown varied tremendously from one isolate to another with MICs ranging from 8 μg/ml to ≥128 μg/ml (the highest level of antibiotic used in the test). The PN MRSA strain is highly resistant to methicillin with an MIC ≥128 μg/ml. The AMSSA strain had an MIC of 4 μg/ml whereas all the other methicillin sensitive strains tested had MICs to methicillin of 2 μg/ml.

2.4.2 COLONY MORPHOLOGY

S.aureus isolates could be divided into three distinct groups based upon their colony morphology.

The first group always produced relatively large colonies greater than 2 mm in diameter (Figure 2.1). All the methicillin sensitive strains (MIC = 2 μg/ml) (except the AMSSA strain) belonged to this group.

The second group always produced smaller colonies no larger than 1.5mm in diameter (Figure 2.2). Most of the methicillin resistant strains tested belonged to this group. This group also included the AMSSA strain (Figure 2.3a) (the only methicillin sensitive strain to produce small colonies).

The third group (represented only by one isolate, the PN MRSA) produced two colony types. Both types were very small measuring 0.8 mm and 0.4 mm in diameter respectively (Figure 2.3b). When either of these colony types were restreaked onto a fresh BHI plate the same diphasic pattern of growth was observed. This occurred even with repeated subculturing of either colony type.

2.4.3 GROWTH RATES

The growth rates (measured as aborsorbance (600 nm) over time) of the PN MRSA, the AMSSA strain and other methicillin resistant and sensitive strains are shown in Table 2.4 and Graph 2.1.
All of the isolates tested had similar growth rates in BHI broth.

2.5 DISCUSSION

In general, there seems to be a good correlation between an isolates colony morphology and its MIC of methicillin. Methicillin sensitive strains (MIC ≤4 μg/ml) produce large colonies (>2 mm in diameter), while methicillin resistant strains (MIC ≥8 μg/ml) produce smaller colonies (<1.5 mm in diameter).

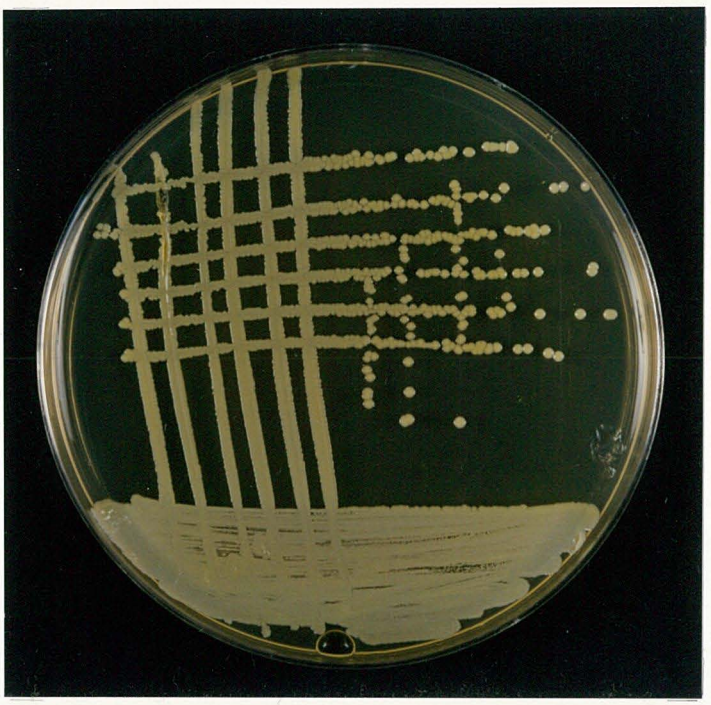
Palmerston North Hospital supplied the AMSSA strain with the comment that it produced colonies typical of MRSA strains. The above results confirm this observation and show that this trait is atypical for a methicillin sensitive isolate.

It is interesting and perhaps surprising to note that the colony size on agar shows no correlation with the growth rate in broth. The reason for this lack of correlation was not investigated, but implies that any extrapolation from agar plates to the growth rate *in vivo* may be in valid because the *in vivo* environment may be more similar to a broth rather than an agar culture.

Table 2.3.
Minimum Inhibitory Concentration of Methicillin for *S.aureus* isolates

Isolate	MIC (μg/ml)	Isolate	MIC (μg/ml)
PN MRSA	≥128	ST90 380	64
AMSSA	4	ST89 580	≥128
WN MRSA	≥128	ST1302	≥128
MRSAT	≥128	SA1	2
MRSAB	16	SA2	2
MRSAG	16	SA4	2
ST90 410	8	SA201	2
ST90 371	16	SA203	2
ST90 236	8	SA209	2
ST90 211	32	SA214	2
ST90 176	32	SA217	2
ST90 103	16	SA301	2
ST88 1377	16	SA303	2
ST90 267	32	SA304	2
AS89 103	4	SA312	2
AS89 105	4		

A



B

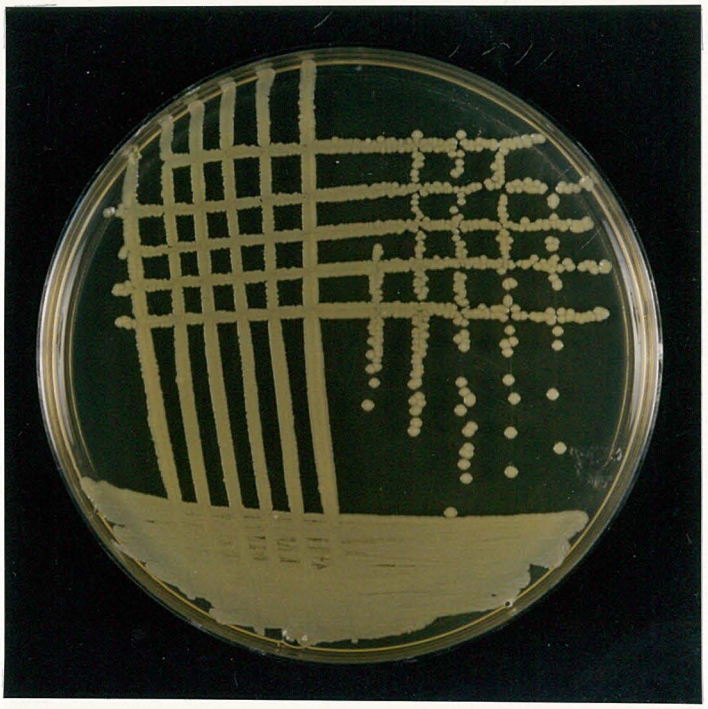
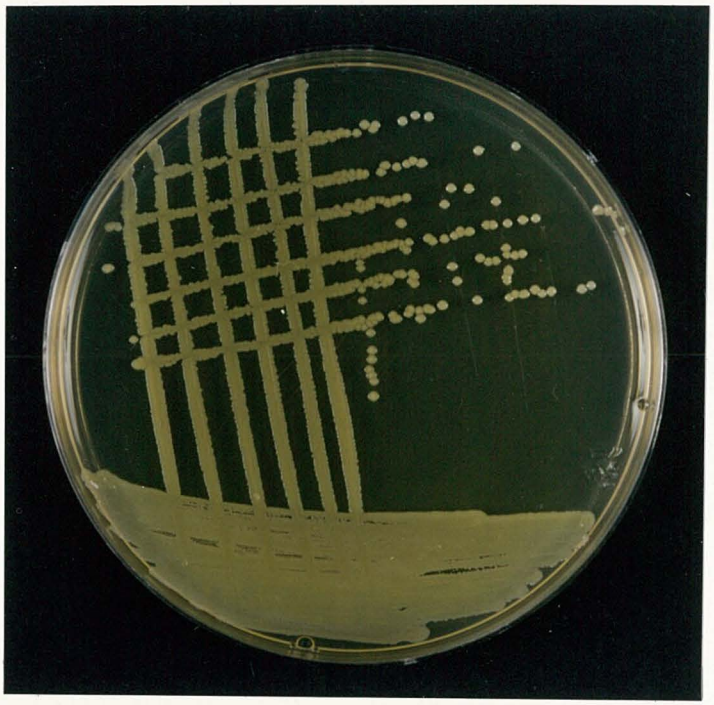


Figure 2.1. Colony morphology of methicillin sensitive *S.aureus* isolates a) SA1 and b) SA4. (Colonies ≥2 mm in diameter).

A



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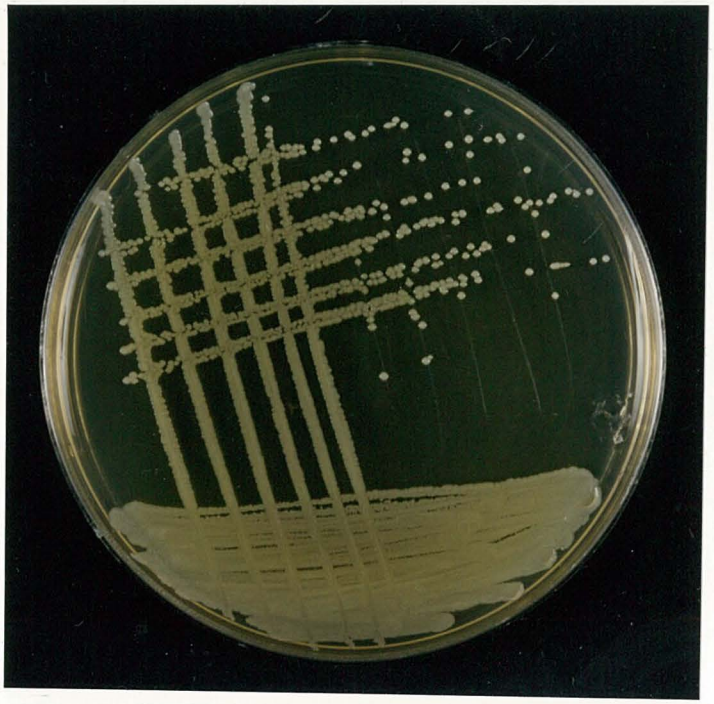
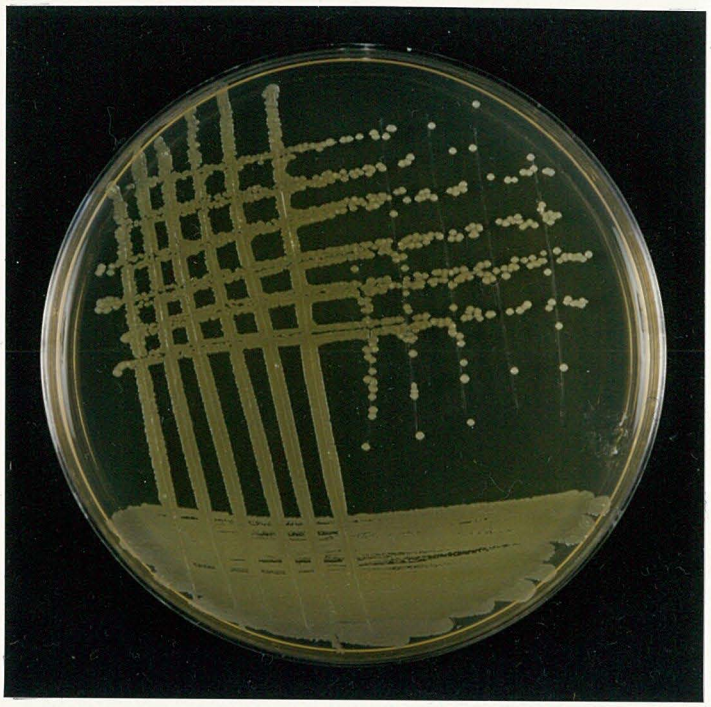


Figure 2.2. Colony morphology of methicillin resistant *S.aureus* strains a) WN MRSA and b) MRSAT. (Colonies ≤ 1.5 mm in diameter).

A



B

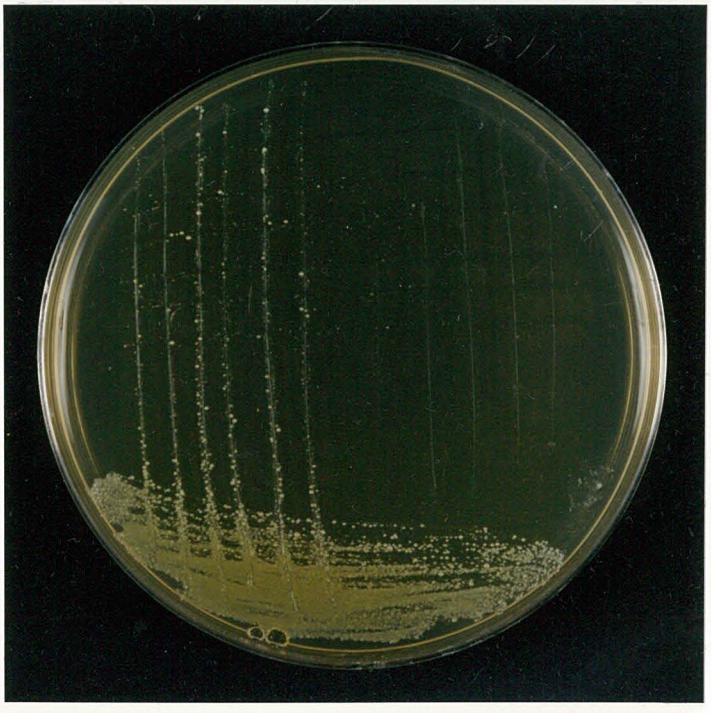
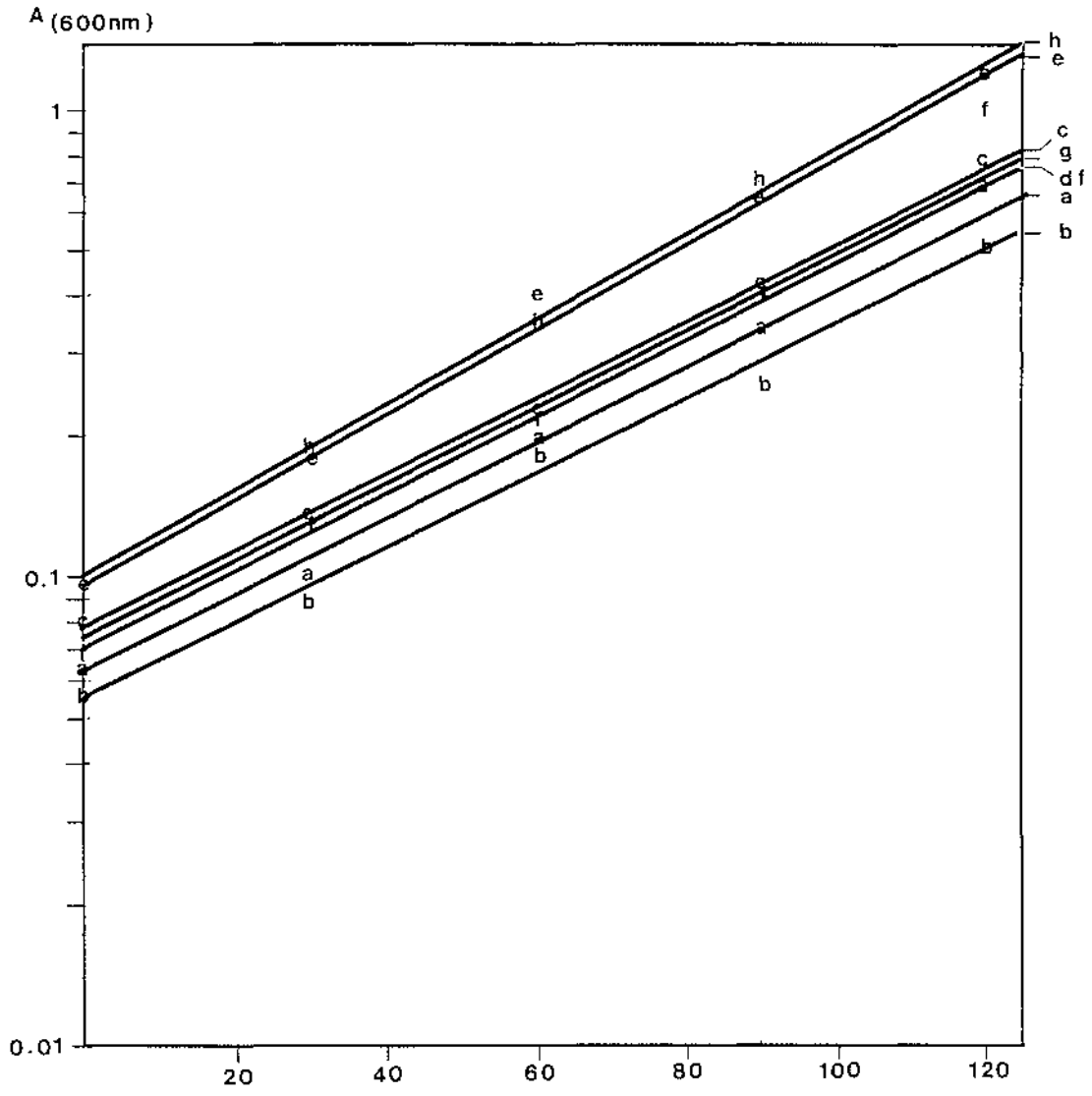


Figure 2.3. Colony morphology of the a) AMSSA strain (colonies 1.5 mm in diameter) and the b) PN MRSA strain (Colonies 0.4 and 0.8 mm in diameter).

Table 2.4.
Absorbance versus Time for *S.aureus* isolates

	ABSORBANCE (600 nm)							
TIME	PN MRSA	AMSSA	WNMRSA	MRSAB	MRSAG	SA1	SA2	SA4
0	0.064	0.056	0.079	0.067	0.098	0.068	0.072	0.093
30 min	0.10	0.89	0.14	0.11	0.18	0.13	0.13	0.19
60 min	0.19	0.18	0.23	0.30	0.40	0.22	0.23	0.35
90 min	0.33	0.26	0.43	0.41	0.65	0.42	0.40	0.7
120 min	0.68	0.5	0.78	0.8	1.2	1.0	0.78	1.2
375 min	2.9	1.8	3.2	3.3	3.9	3.5	2.6	3.2
23 hrs	5.9	5.2	6.3	7.3	7.2	6.6	4.1	5.4

Graph 2.1 Absorbance versus Time for *S.aureus* isolates



KEY

a	PN MRSA	d	MRSAG
b	AMSSA	e	SA1
c	WN MRSA	f	SA2
d	MRSAB	g	SA4

CHAPTER 3
PROBING OF *S.AUREUS* DNA FOR THE *MEC* GENE

3.1 INTRODUCTION

Do all MRSA with a MIC ≥8 μg/ml possess the *mec* gene?

MRSA isolates recovered from a range of countries throughout the world are typically resistant to high levels of methicillin (ie ≥64 μg/ml). These strains are typically multiply resistant (Lyon and Skurray 1987, Heffernan *et al* 1993). Such strains have been isolated in New Zealand (isolates 11 to 16 in Table 3.1, pg 29) and include the Palmerston North and Wellington outbreak MRSA strains (PN MRSA and WN MRSA). However the majority of MRSA strains isolated in New Zealand in recent years are resistant to only low levels of methicillin (ie 8 to 32 μg/ml), and, unlike the MRSA found overseas, are not generally multiply resistant (CDNZ suppl.1 1992) (isolates 2 to 8 Table 3.1).

In multiply resistant MRSA strains the *mec* gene and many of the other resistance determinants are found on the chromosome as part of a large '*mec* gene complex' (up to 50 kilobases in size) (Dubin *et al* 1992). The 'low level singularly resistant' MRSA strains found in New Zealand must lack that part of the '*mec* gene complex' that confers resistance to antibiotics other than methicillin and, as they are resistant to only low levels of methicillin it is possible that they also lack the *mec* gene itself. If so, some other mechanism besides the possession of a gene coding for PBP2a, may mediate the low level of methicillin resistance shown by these isolates.

The presence or absence of the *mec* gene in these 'low level singularly resistant' and 'high level multiply resistant' MRSA strains is investigated in this chapter using a DNA probe specific for the *mec* gene.

Does the AMSSA strain possess the *mec* gene ?

As the AMSSA strain was isolated from a patient who had been infected with the PN outbreak MRSA strain and, as has it had the colony morphology typical of a methicillin resistant strain the medical microbiologist at Palmerston North Hospital was concerned that this strain may have been derived *in vivo* from the PN MRSA, perhaps by an easily reversible mutation (such as a point mutation or small deletion) and if so may rapidly revert to resistance if exposed to methicillin. To investigate whether the AMSSA strain possessed the *mec* gene it was probed with a PBP2a gene fragment. This was done in parallel with the PN MRSA strain.

Table 3.1.
Antimicrobial Susceptibility of *S.aureus* isolates
(provided by M. Brett, NZCDC)

No.	Isolate	Methicillin MIC µg/ml	Antimicrobial Resistance
1	ST90 410	8	Me, Pn, Em, Gm
2	ST90 371	16	Me, Pn
3	ST90 236	8	Me, Pn
4	ST90 211	32	Me, Pn
5	ST90 176	16	Me, Pn
6	ST90 103	8	Me, Pn, Em
7	ST88 1377	16	Me, PN,
8	ST90 267	16	Me, Pn
9	AS89 103	4	Ox, Pn
10	AS89 105	4	Ox, Pn
11	ST90 380	64	Me, Pn, Em, Co
12	WN MRSA	≥128	Me, Pn, Cm, Em, Co
13	ST89 580	≥128	Me, Pn, Cm, Em, Gm, Co
14	ST89 1302	≥128	Me, Pn, Em, Gm
15	ST88 988	64	Me, Pn, Em, Gm
16	PN MRSA	≥128	Mn, Em, Gm, Co
17	AMSSA	4	Pn, Em, Co

Antimicrobials Tested:

Methicillin	(Mn)
Penicillin	(Pn)
Chloramphenicol	(Cm)
Erythromycin	(Em)
Fusidic acid	(Fa)
Gentamycin	(Gm)
Vancomycin	(Vm)
Cotrimoxazole	(Co)
Rifampicin	(Rf)
Mupirocin	(Mu)

3.2 MATERIALS

Commonly Used Reagents

0.2 M EDTA	
EDTA (disodium salt)	7.44 g
pH to 8.0 with NaOH	
Distilled water to	100.0 ml

1 M Tris-HCL (pH 8.0)	
Trizma Base (Sigma)	12.11 g
pH to 8.0 with HCl	
Distilled water to	100.0 ml

5 M NaCl	
NaCl	29.22 g
Distilled water to	100.0 ml

Extraction of *S.aureus* DNA

Lysis of *S.aureus* cells

Lysostaphin 1 mg/ml	
Lysostaphin (Sigma)	5 mg
Sterile distilled water to	5.0 ml

Lysis Buffer (pH 8.0)	
1 M Glucose	2.5 ml
0.2 M EDTA	2.5 ml
1 M Tris-HCL (pH 8.0)	1.25 ml
5 M NaCl	1.5 ml
Distilled water to	50.0 ml

Sodium dodecyl sulphate (SDS) 10%	
SDS (Sigma)	10.0 g
Distilled water to	100.0 ml

Pronase Type XIV 10 mg/ml	
Pronase (Sigma)	0.2 g
Sterile distilled water to	20.0 ml

The solution was preincubated at 37°c for three hours to self-digest contaminants, especially DNase activity. This was then stored at -20°c.

RNase 10 mg/ml

RNase - from bovine pancreas (Sigma)	0.1 g
Sterile distilled water to	10.0 ml

This solution was preincubated at 90°c for 10 minutes to destroy DNase activity. It was then stored at -20°c.

5 M Sodium perchlorate

Sodium perchlorate	61.22 g
Distilled water to	100.0 ml

DNA Extraction

TE Buffer (Tris-EDTA)

1 M Tris-HCL (pH 7.5)	10.0 ml
0.2 M EDTA pH7.5	5.0 ml
Distilled water to	1000.0 ml

Phenol/chloroform/isoamyl alcohol solution
A 25:24:1 solution, respectively, was prepared.
1/10 the volume of STE Buffer was then bubbled through the mixture.

Preparation of Slot Blots

20x Standard Saline Citrate (SSC) Stock Solution

NaCl	175.3 g
Sodium citrate	88.2 g
Distilled water to	1000.0 ml

0.4 M NaOH

NaOH	1.6 g
Distilled water to	100.0 ml

2 M Ammonium acetate (pH 7.0)

Ammonium acetate	15.4 g
adjust pH to 7.0	
Distilled water to	100.0 ml
Store at 4°c	

***E.coli* Plasmid Preparation**

Luria Broth

NaCl	5 g
Tryptone (Bacto)	10 g
Yeast extract (Difco)	5 g
pH to 7-7.5 with 5 M NaOH	0.5 ml
Distilled water to	1000.0 ml

Luria Agar	
NaCl	5 g
Tryptone	10 g
Yeast extract	5 g
pH to 7-7.5 with 5 M NaOH	0.5 ml
Agar	15 g
Distilled water to	1000.0 ml

Ampicillin 5 mg/ml	
Ampicillin	0.05 g
Distilled water to	10.0 ml

Luria Broth + Ampicillin (100 µg/ml)	
Luria Broth	4.9 ml
Ampicillin (5 mg/ml)	0.1 ml

Luria Agar + Ampicillin (110 µg/ml)	
Luria Agar	196.0 ml
Ampicillin (5 mg/ml)	4.0 ml

Melt agar and equilibrate to 50°c before adding ampicillin

Solution I	
Glucose	4.95 g
1 M Tris-HCl (pH 8.0)	12.5 ml
0.2 M EDTA (disodium salt)	25.0 ml
Distilled water to	500.0 ml

Autoclave at 121°c for 15 minutes

Solution II (prepare fresh)	
NaOH	0.8 g
SDS	1.0 g
Distilled water to	100.0 ml

Solution III	
Potassium acetate	29.44 g
Glacial acetic acid	11.5 ml
Distilled water to	100.0 ml

Lysozyme 50 mg/ml (prepare fresh)	
Lysozyme	0.05 g
Distilled water to	1.0 ml

Megaprime DNA Labelling Protocol

Megaprime DNA Labelling Kit (Amersham)

Primer solution

Random nonamer primers in an aqueous solution

Megaprime reaction Buffer
dATP, dGTP, and dTTP in Reaction buffer.

Reaction Buffer
A concentrated reaction buffer containing:
Tris-HCl (pH7.5)
MgCl
2-mercaptoethanol

Enzyme Solution
1 unit/µl DNA polmerase I 'Klenow' fragment (cloned) in:
50 mM potassium phosphate (pH 6.5)
10 mM 2-mercaptoethanol
50% glycerol

Hybridisation Buffer

1M Hepes Buffer (pH 7.0)	25.0 ml
20x SSC	75.0 ml
Herringsperm DNA (3 mg/ml)	3.0 ml
20% SDS	2.5 ml
Ficoll (Sigma)	1.0 g
Bovine Serum Albumin (Sigma)	1.0 g
Polyvinyl pyrrolidone (PVP-10 Sigma)	1.0 g
Distilled water to	500.0 ml

Store at 4°c
Warm to 37°c before use

DIG Labeling and Detection Kit (Boeringer Mannheim Biochemica)

Hybridisation
Hexanucltotide Mixture (DIG kit)
10x concetrated hexanucleotide mixture

dNTP Labeling Mixture (DIG kit)
10x concentrated dNTP labeling mixture containing:
1 mmol/l dATP
1 mmol/l dCTP
1 mmol/l dGTP
0.65 mmol/l dTTP
0.35 mmol/l Dig-dUTP
pH 6.5 (20°c)

Klenow Enzyme, labeling grade, 2 U/µl (DIG kit)

4 M LiCl

LiCl	1.7 g
Distilled water to	10.0 ml

Hybridistaion Solution

20xSSC	62.5 ml
10% N-lauroyl sarcosine	2.5 ml
10% SDS	500 µl
Blocking reagent (DIG kit)	1.25 g
Sterile distilled water	184.5 ml

Washing the Membrane

Wash Solution I

20xSSC	40 ml
10% SDS	4 ml
Distilled water to	400 ml

Wash Solution II

20x SSC	2 ml
10% SDS	4 ml
Distilled water to	400 ml

Colour Detection

Buffer 1

Trisma Base	12.1 g
NaCl	8.77 g
Distilled water to	1000.0 ml

Buffer 2

Blocking reagent (DIG kit)	2.5 g
Buffer 1	500.0 ml

Prepare 1 hour in advance and dissolve at 50-70°c (Buffer remains turbid).

Buffer 3

Trisma Base	3.0 g
NaCl	1.46 g
$MgCl_2$	1.19 g

pH to 9.5 with NaOH
Distilled water to 250.0 ml

Anti-digoxigenin alkaline phosphatase conjugate (DIG kit)
750 U/ml polyclonal sheep anti-digoxigenin Fab-fragments conjugated to alkaline phosphatase

antibody-conjugate (150 U/ml)

anti-digoxigenin-AP conjugate	4 µl
Buffer 1	20.0 ml

NBT-solution (DIG kit)
75 mg/ml nitroblue tetrazolium salt in dimethylformamide (70% v/v)

X-Phosphate-solution (DIG kit)
50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in dimethylformamide

Colour Solution

NBT-solution	45 µl
X-Phosphate-solution	35 µl
Buffer 3	10 ml

Prepare fresh.

3.3 METHODS

3.3.1 EXTRACTION OF *S.AUREUS* DNA

Lysis of *S.aureus* cells

A 10 µl aliquot of lysostaphin (1 mg/ml) was added to 190 µl of Lysis buffer (Archer and Pennell 1990). A loopful of bacteria, obtained from heavy growth on a BHI agar plate was then added to the mixture and incubated for 1 hour at 37°C. 150 µl of 10% SDS was then added, and the bacterial suspension incubated at 50°C for 10 minutes. Following this 10 µl of pronase (10 mg/ml) was added and the lysate was incubated at 50°C overnight to ensure that the cells were lysed and that their proteins digested. 10 µl of RNase (10 mg/ml) was then added and incubated for 1 hour at 50°C. 90 µl of 5 M sodium perchlorate was added and the lysate was incubated at 50°C for a further 60 minutes.

DNA Extraction

140 µl of TE buffer was added to the *S.aureus* cell lysate. An equal volume (600 µl) of phenol/chloroform/isoamyl alcohol solution then added, mixed by inversion, and centrifuged in an eppendorf microfuge for 30 minutes. The upper aqueous layer containing the DNA was placed in another centrifuge tube and an equal volume of chloroform was added, mixed by inversion, and centrifuged for 30 minutes. The upper aqueous layer was again removed to a clean centrifuge tube and 1/20 the volume of 5 M NaCl and two volumes of cold (-20°C) absolute ethanol were added. The solution was placed at -20°C overnight to precipitate the DNA. The solution was then centrifuged for 30 minutes in an eppendorf microfuge and the supernatant discarded. The pellet was washed with 1 ml of 70% ethanol, dried in a speedvac for 15 minutes, and then resuspended in 15 µl of sterile TE buffer.

3.3.2 PREPARATION OF SLOT BLOTS OF *S.AUREUS* DNA

Three sheets of Bio-Dot Format Filter paper were washed in 6xSSC and placed in a Slot-Blot apparatus (Biorad). A nylon membrane, cut slightly bigger, was then placed on top of the filter paper, and the apparatus was screwed together as tightly as possible. 15 µl of *S.aureus* DNA was denatured by incubation in 100 µl of 0.4 M NaOH for 5-10 minutes, neutralized by the addition of 100 µl of cold (4°C) ammonium acetate solution and added to the wells under gentle vacuum (tap open). Once all the liquid had been drawn through (1-2 hours) each slot was washed with 200 µl of 2x SSC, drawn through under full vacuum (Tap closed). The apparatus was then disassembled and the nylon membrane was rinsed in 2x SSC, placed on a piece of filter paper to dry, and baked for 2 hours at 80°C in a vacuum oven.

3.3.3 ISOLATION OF THE pUC18 PLASMID FROM *E.COLI*

Two colonies of *E.coli* strain PG0164, obtained from growth on a LB Amp agar plate, were inoculated into 5 ml of LB Amp broth and incubated on a rotary shaker at 37°C overnight. 1.5 ml of culture was then placed into two eppendorf tubes and centrifuged for 1 minute to pellet the cells. The supernatant was discarded and the cells resuspended in 100 µl of Solution I. 10 µl of lysozyme (50mg/ml) was added, the solution was mixed by vortexing, and left to stand at room temperature for 5 minutes to lyse the cells. 200 µl of Solution II was then added, mixed by inversion, and placed on ice for 5 minutes. 150 µl of Solution III was added, mixed briefly by vortexing and left on ice for 5 minutes to precipitate proteins and chromosomal DNA. The lysate was then centrifuged for 3 minutes and the supernatant put into clean eppendorf tube. 0.6 volumes of isopropanol was added and left at room temperature for 2 minutes to precipitate the plasmid DNA. The mixture was then centrifuged for 2-3 minutes and the supernatant discarded. The pellet, containing the plasmid DNA was washed with 1 ml 70% ethanol, dried in a speedvac for 20 minutes, and resuspended in 25 µl of sterile water.

3.3.4 LINEARISATION OF PLASMID DNA WITH *Eco*R1

5 µl of plasmid DNA was added to an eppendorf tube containing 2 µl of 10x H Buffer (Promega) and 11 µl of sterile distilled water. 2 µl of the restriction enzyme *Eco*R1 (Promega) was then added, mixed by brief centrifugation, and incubated at 37°c for 90 minutes.

The reaction mixture was then stored at 4°c until required for use as a probe for the PBP2a gene.

3.3.5 LABELLING THE PBP2A GENE PROBE

The *E.coli* strain PG0164 containing the plasmid pUC18 plus the cloned 1.1 kilobase *Bgl*II-*Xba*I PBP2a gene fragment (Archer and Pennell 1990) was kindly provided by Dr Gordon Archer of the Medical College of Virginia.

General approach

The Probe was labelled with either a radioactive (^{32}P) or nonradioactive (Boehringer Mannheim) detector. The ^{32}P-labelled nucleotide (dCTP) was incorporated into probe DNA by random hexamer priming using the Megaprime DNA labelling kit (Amersham). The nonradioactive label, digoxigenin-dUTP, was incorporated into probe DNA by random hexamer priming using the Boehringer-Mannheim digoxigenin labelling kit. In both cases the entire plasmid, pUC18 plus the cloned 1.1 kb PBP2a gene fragment, was labelled for use as a probe as *E.coli* vector sequences have been shown not to hybridize with staphylococcal DNA (Archer and Pennell 1990).

LABELLING THE PROBE USING RADIOACTIVE ^{32}P

Megaprime DNA Labelling Protocol

5 µl of *Eco*R1-cut pUC18 plasmid DNA containing the 1.1 kb PBP2a gene fragment was added to 5µl of primer solution and 25 µl of sterile distilled water in an eppendorf tube. The DNA was then denatured by heating for 5 minutes at 95-100°c.

10 µl of Megaprime reaction buffer and 5 µl of Reaction buffer were then added. This was followed by 3µl of radiolabelled dCTP and then 2 µl of enzyme solution (the Klenow fragment of DNA polymerase I). The tube was capped and incubated at 37°c for 5-10 minutes. The reaction was stopped and the DNA denatured by heating the solution to 95-100°c for 5 minutes.

Measuring the amount of [^{32}P]dCTP incorporated into the probe DNA

2 µl of labelled DNA solution was spotted onto one end of a strip of plastic-backed filter paper and left to dry. The filter paper was then placed vertically, with the DNA at the bottom, in a vial containing a small amount of 2 M HCl (free [^{32}P]dCTP travels up the filter paper with the solvent while [^{32}P]dCTP incorporated into DNA remains at the bottom of the filter paper). When the solvent front had travelled up the filter paper the paper was cut in half and each half was placed into an empty vial. The radioactivity of the top and bottom halves of the filter paper was then measured in disintegrations/minute using a scintillation counter. About 50% of the [^{32}P]dCTP should and did become incorporated into the plasmid DNA.

Prehybridisation

The baked nylon membrane blotted with *S.aureus* DNA was placed in a Hybridisation tube and 15 ml of Hybridisation buffer was added. The tube was rotated to wet the membrane evenly and then placed in a horizontal rotator in a 65°c incubator for 2 hours.

Hybridisation

48 µl of ^{32}P labelled probe solution was added to the tube containing the Hybridisation buffer and the nylon membrane. The Hybridisation tube was then placed back in the horizontal rotator and incubated at 65°c overnight.

Washing the membrane

The Hybridisation buffer was discarded. 2x SSC was added to the tube to wash the membrane and to work it down to the mouth of the tube. The 2xSSC was then discarded, and using forceps and gloves the nylon membrane was transferred to an

icecream-box containing 100 ml of 2x SSC . This was shaken for 15 minutes at room temperature on a reciprocating shaker. The 2x SSC was then discarded and the above step repeated with 2x SSC and 0.1x SSC.

Detection of ^{32}P using Autoradiography

The nylon membrane was then mounted on Whatman 3MM filter paper, covered with gladwrap, and placed in an 18 x 24 cm cassette. Kodak film was placed over the nylon membrane in the dark room and an intensifying screen was placed over the film and under the filter paper. The cassette was then placed at -70°c for 24 hours before developing.

LABELLING THE PROBE USING NONRADIOACTIVE DIGOXIGENIN-dUTP

Principle

DNA is labelled by random primed incorporation of digoxigenin-labelled deoxyuridine-triphosphate. The dUTP is linked via a spacer-arm to the steroid hapten digoxigenin (Dig-dUTP). After hybridisation to the target DNA the hybrids are detected by enzyme-linked immunoassay using an antibody-conjugate (anti-digoxigenin alkaline phosphatase conjugate) and a subsequent enzyme-catalyzed colour reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-Phosphate) and nitroblue tetrazolium salt (NBT) (DIG kit manual).

Digoxigenin-dUTP Labelling Protocol

5 µl of *Eco*R1 cut pUC18 plasmid DNA containing the 1.1 kilobase PBP2a gene fragment was added to 5 µl of sterile water in an eppendorf tube and denatured by heating for 10 minutes at 95°c in a heating block. Immediately following this the eppendorf tube was plunged into ice water to prevent reannealing and then placed on ice. 2 µl of hexanucleotide mixture, 2 µl of dNTP labelling mix, 5 µl of sterile milli-Q water, and 1 µl of klenow enzyme (2 U/µl) were then added and the mixture was incubated at 37°c for 60 minutes. The reaction was stopped by the addition of 2 µl of 0.2M EDTA and 2 µl of 4 M LiCl. 60 µl of cold (-20°c) absolute ethanol was

then added and the mixture placed at -20°c overnight to precipitate the DNA. The solution was then centrifuged for 15 minutes at 4°c in an eppendorf microfuge and the supernatant discarded. The labelled DNA pellet was then washed with 200 µl of cold 70% ethanol, dried in a speedvac for 10 minutes and resuspended in 50 µl of sterile TE buffer.

Prehybridisation

The baked nylon membrane blotted with *S.aureus* DNA was placed in a Hybridisation tube and 20 ml of Hybridisation solution were added. The tube was rotated to wet the membrane and then placed in a horizontal rotator in a 65°c incubator for 2 hours

Hybridisation

50 µl of digoxigenin-dUTP labelled DNA solution was denatured by heating at 95°c for 10 minutes in a heating block. The solution was then plunged into ice water to prevent reannealing and added to the tube containing the Hybridisation solution and the nylon membrane. The Hybridisation tube was then placed back in the horizontal rotator and incubated at 65°c overnight

Washing the Membrane

The hybridisation solution was discarded, 200 ml of Wash Solution I was added and the hybridisation tube placed on a horizontal rotator for 5 minutes at room temperature. Wash Solution I was then discarded and the above step repeated. 200 ml of warm (65°c) Wash Solution II was then added and the tube placed in a horizontal rotator in a 65°c incubator for 15 minutes. This step was repeated.

Colour Detection

Using gloves and forceps the nylon membrane was removed from the hybridisation tube and placed into 100 ml of Buffer 1 and shaken for 1 minute on a reciprocating shaker. Buffer 1 was discarded and 100 ml of Buffer 2 was added and shaken for 30 minutes at room temperature. The nylon membrane was again washed briefly in 100 ml of Buffer 1 and then placed in a plastic bag. The antibody-conjugate was diluted

to 150 MU/ml by adding 4 µl to 20 ml of Buffer 1 and was then added to the bag containing the nylon membrane. The bag was sealed and incubated at room temperature for 30 minutes. The nylon membrane was then removed from the bag and placed in 100 ml of Buffer 2 and shaken for 15 minutes at room temperature to remove any unbound antibody/conjugate. This step was repeated and then the membrane was placed in 20 ml of Buffer 3 for 2 minutes. The nylon membrane was then placed in another plastic bag and 10 ml of Colour Solution was added. The bag was sealed and placed in the dark until the test-negative *S.aureus* strain just began to show colour (about 10 minutes). The colour reaction was then stopped by placing the membrane into 100 ml of TE buffer.

3.4 RESULTS

The 1.1 kb PBP2a gene probe was labelled with either ^{32}P or nonradioactive digoxigenin-dUTP and hybridised to *S.aureus* DNA on a nylon membrane (Table 3.1, pg 44). The results using the ^{32}P labelled mec gene probe are shown in Figure 3.1a. The results using the digoxigenin-dUTP labelled *mec* gene probe are shown in Figure 3.1b.

The results using either probe were in total agreement.

All the 'high level multiply resistant' MRSA strains (MIC ≥64 µg/ml) hybridised with the *mec* gene fragment (lanes 2E to 4A). This included the PN MRSA strain (lane 4A).

All the 'low level singularly resistant' MRSA strains (MICs 8 to 32 µg/ml) hybridised the *mec* gene probe (lanes 1A to 2B).

The two 'borderline methicillin resistant' strains (defined by their ability to grow in 4 µg/ml oxacillin (resistant (NCCLS 1990)) but inability to grow in 8 µg/ml of methicillin (sensitive (NCCLS 1990)) did not hybridise with the *mec* gene fragment (lanes 2C and 2D).

None of the methicillin sensitive strains (MIC ≤4 µg/ml) hybridised with the 1.1 kb PBP2a gene fragment (lanes 4C to 4F). The AMSSA strain (MIC 4 µg/ml), isolated from the same patient as the PN MRSA, also did not hybridise with the 1.1 kb

PBP2a gene fragment (Lane 4B).

3.5 DISCUSSION

All of the 'low level singularly resistant' MRSA strains (MIC 8 to 32 μg/ml) hybridised with the mec gene fragment, which implies that the *mec* gene is responsible for resistance to methicillin in these isolates.

The two 'borderline' MRSA strains did not hybridise with the *mec* gene showing that the *mec* gene is not responsible for the low level of resistance to oxacillin in this group.

In general, there was a good (in fact perfect) correlation between the MIC at which a strain is considered resistant (≥8μg/ml) and possession of the *mec* gene. As all of the *S.aureus* strains with MICs ≥8 μg/ml hybridised with the *mec* gene, while all of the *S.aureus* strains with MICs ≤4 μg/ml did not hybridise with the *mec* gene probe.

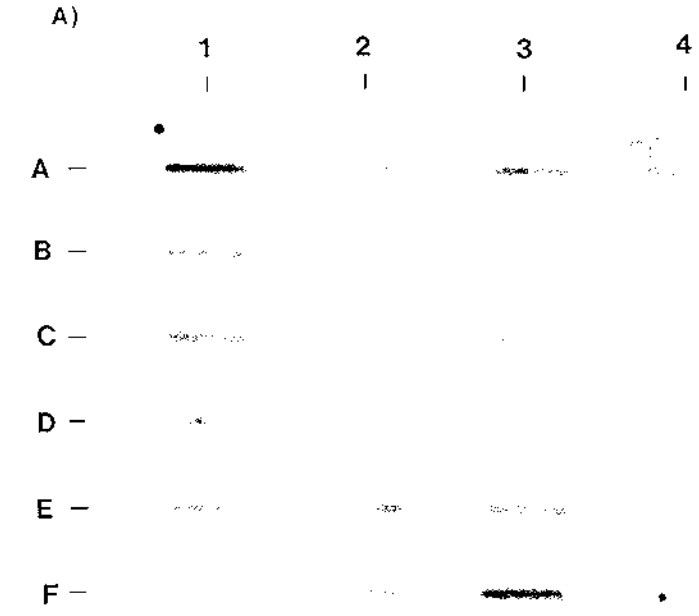
The AMSSA strain (MIC = 4 μg/ml) also did not hybridise with the PBP2a gene fragment. The 1.1 kb PBP2a gene probe covers most of the structural sequence for the PBP2a gene. Therefore if the AMSSA strain was derived *in vivo* from the PN MRSA its sensitivity was not due to an easily reversible point mutation or to a small deletion, but rather must be due to the deletion of at least 1.1 kilobases which covers most of the coding region for PBP2a. It follows from this that the AMSSA strain is no more likely than any other methicillin sensitive strain to develop resistance to methicillin following exposure to the drug.

Deletion of such a large amount of DNA is not improbable as all methicillin resistant strains that have been converted to sensitivity *in vitro* to date have lost the entire *mec* gene plus all or part of the *mec* gene complex. The size of the deletion in these strains varied considerably but could be up to 200 kb in size. In most cases one or both of the deletion endpoints occurred at or near an insertion sequence or a transposon attachment site (Inglis *et al* 1990, Wada *et al* 1991).

The remainder of my thesis investigates the possibility that the AMSSA strain was derived from the PN MRSA by comparing the two strains using a variety of techniques.

Table 3.2.
Order of *S.aureus* isolates on the Nylon membrane

	Lane			
Lane	1	2	3	4
A	ST90 410	ST88 1377	ST89 580	PN MRSA
B	ST90 371	ST90 267	ST89 1302	AMSSA
C	ST90 236	AS89 103	ST88 988	SA1
D	ST90 211	AS89 105	MRSAT	SA2
E	ST90 176	ST90 380	MRSAG	SA4
F	ST90 103	WN MRSA	MRSAB	SA301



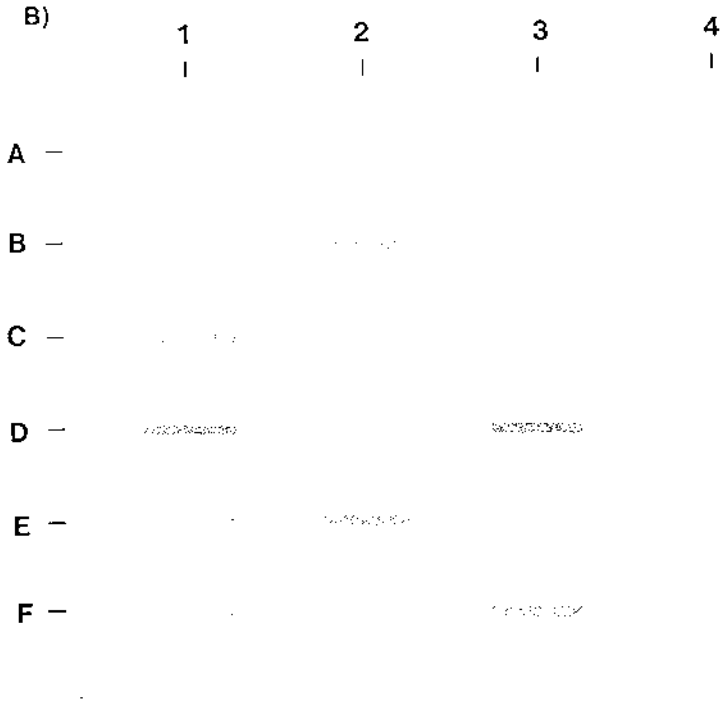


Figure 3.1. Hybridisation of *S.aureus* DNA with a) ^{32}P-labelled or b) digoxigenin-dUTP labelled *mec* gene probe.

CHAPTER 4
COMPARISON OF THE AMSSA STRAIN WITH THE PN MRSA AND OTHER METHICILLIN RESISTANT AND SENSITIVE ISOLATES

4.1 INTRODUCTION

This chapter investigates whether the AMSSA strain was derived *in vivo* from the PN MRSA by comparing the two isolates with other methicillin resistant and sensitive staphylococci using phage typing (with and without heat shock), reverse phage typing, plasmid profiles, and restriction endonuclease analysis.

4.2 MATERIALS

Phage Typing and Reverse Phage Typing

Typing Broth	
Difco Nutrient Broth	20 g
NaCl	5 g
Adjust pH to 7.2 - 7.4	
Distilled water to	1000 ml

Standard Typing Agar	
Oxoid Nutrient Broth No.2	120 g
NaCl	30 g
Distilled water (100°c)	6000 ml
Oxoid Agar No.1	42 g
1% $CaCl_2$	240 ml

Adjust pH to 7.4 - 7.6
Dispense into 200 ml aliquots and autoclave for 15 minutes at 121°c.

Preparation of *S.aureus* DNA

Lysis of *S.aureus* bacteria

Luria Glucose Broth plus Tris-HCl	
NaCl	4.0 g
Tryptone (Bacto)	8.0 g
Yeast Extract (Difco)	4.0 g
Glucose (0.2% W/V)	1.6 g
pH to 7-7.5 with 5M NaOH	0.4 ml
1M Tris-HCl (pH 7.5)	16.0 ml
Distilled water to	800.0 ml

TES (Tris-EDTA-Saline)	
1M Tris-HCl (pH 7.8)	15.0 ml
5M NaCl	5.0 ml
0.2M EDTA	12.5 ml
Distilled water to	500.0 ml

TES plus 2.5M NaCl

1M Tris-HCl (pH 7.8)	15.0 ml
0.2M EDTA	12.5 ml
NaCl	73.05 g
Distilled water to	500.0 ml

Lysostaphin 1mg/ml

Lysostaphin (Sigma)	5 mg
Sterile distilled water to	5.0 ml

10% Sarkosyl

Sarkosyl	10 g
Distilled water to	100.0 ml

0.2 M EDTA

EDTA (disodium salt)	7.44 g
pH to 8.0 with NaOH	
Distilled water to	200.0 ml

RNase 2mg/ml

RNase (Sigma)	0.02 g
Distilled water to	10.0 ml

DNA Extraction

Phenol/Chloroform/Isoamyl alcohol solution
A 25:24:1 solution, respectively, was prepared.
1/10 the volume of STE Buffer was then bubbled through the mixture.

STE (Saline-Tris-EDTA) Buffer

5.0M NaCL	20.0 ml
1.0M Tris-HCl (pH 7.5)	50.0 ml
0.2M EDTA (pH 7.2)	5.0 ml
Distilled water to	100.0 ml

Chloroform/Isoamyl alcohol
A 24:1 solution, respectively was prepared.

3 M Sodium acetate	
Sodium acetate	24.61 g
Distilled water to	100.0 ml

Agarose Gel Electrophoresis

Tris Borate Buffer (10x TBB)	
Trizma Base	108.0 g
EDTA (disodium salt)	9.3 g
Boric Acid	55.0 g
adjust pH to 8.2	
Distilled water to	1000.0 ml

1x TBB	
10x TBB	50.0 ml
Distilled water to	500.0 ml

1% Agarose	
Agarose	0.5 g
1x TBB to	50.0 ml

Bromophenol Blue Dye	
Bromophenol Blue	0.05 g
Glycerol	40.0 ml
Distilled water to	50.0 ml

Ethidium bromide 10 mg/ml	
Ethidium bromide	0.1 g
Distilled water to	10.0 ml
Store in a dark bottle.	

4.3 METHODS

4.3.1 COMPARISON OF *S.AUREUS* ISOLATES USING PHAGE TYPING AND REVERSE PHAGE TYPING

Phage Typing of *S.aureus* isolates

Colonies of isolates to be typed were selected from Blood Agar plates, inoculated into 3 ml of Typing broth and incubated for 5 hours in a 37°c waterbath. Using a sterile pasteur pipette the cultures were flooded onto the surface of Standard Typing agar plates. The excess liquid was removed and the plates were dried at room temperature for 20 minutes. Twenty three *S.aureus* bacteriophage from the Basic International Set (Table 4.1), at 100x Routine Test Dilution, were applied to each plate using a Lidwell Applicator (Blair and Williams 1961). The plates were incubated at 30°c for 18 hours.

Phage Typing of *S.aureus* isolates following Heat Shock

If a culture showed no significant lysis with any phage the isolate was retyped following heat shock.

In this case a single colony was inoculated onto a fresh Blood Agar plate and incubated at 37°c overnight. Two colonies were then inoculated into separate Typing broths and incubated in a 37°c waterbath for 5 hours. One broth was placed in a 55°c waterbath for 3 minutes and then flooded onto a Typing plate, while the other was flooded onto the Typing plate without heat treatment. After drying, the plates were then inoculated with phage using the same phage concentrations and conditions as above.

Table 4.1.
International Basic Set of Phage for Typing *Staphylococcus aureus*
(Blair and Williams 1961)

Lytic Group	Phage Numbers				
I	29	52	52A	79	80
II	3A	3C	55	71	81
III	6	42E	47	53	54
III	75	77	83A	84	85
	94	95	96		

Reverse Phage Typing of *S.aureus* isolates

Indicator strains used were the propagating strains from the international phage set PS 6, 42E, 47, 75, 84, and 85; the Wellington Hospital outbreak MRSA strain, 85653; four methicillin sensitive strains, 86614, 861653, 861671, and 861691; and three reverse phage typing strains W57, 1030, and 18042 (Heffernan *et al* 1993).
Two strains were used as controls: the Wellington Hospital outbreak strain 85653, and the Palmerston North Hospital outbreak strain 85203.

Isolates to be reverse phage typed, controls and indicator strains were streaked for isolated colonies on Blood Agar plates and incubated at 37°c in a 5% CO_2 incubator overnight. All strains were then inoculated into Typing broth and incubated in a 37°c waterbath for 5 hours.
The indicator strain cultures were then flooded onto the surface of Typing plates and dried at room temperature for 20 minutes.
The isolates and control strains were centrifuged for 10 minutes at 3000 rpm and the supernatants, containing the bacteriophage, placed in a well of the Reverse Typing Block (Blair and Williams 1961). These supernatants were applied to each of the indicator strains using a Lidwell Applicator and the plates incubated at 30°c for 18 hours.

4.3.2 PREPARATION OF DNA FROM *S.AUREUS* STRAINS FOR PLASMID ANALYSIS AND RESTRICTION ENDONUCLEASE DIGESTION

Lysis of *S.aureus* cells

The lysis procedure follows the Australian National University method (Matthews, Reed and Stewart 1987) with a few modifications.

Two to four *S.aureus* colonies, obtained from a BHI agar plate, were inoculated into 20 ml of LG broth plus Tris-HCl and incubated at 37°C overnight with shaking. The

overnight culture was centrifuged for 5 minutes at 12000 g and the pellet washed twice in 10 ml of TES and resuspended in 2.5 ml of TES plus 2.5 M NaCl. 100 µl of lysostaphin (1 mg/ml) was added and the solution incubated for 90 minutes at 37°C. 750 µl of 10% sarkosyl and 630 µl of 0.2 M EDTA were then added and the lysed suspension was incubated at 65°C for 20 minutes. 100 µl of RNase (2 mg/ml) was added and incubated for 1 hour at 50°C. 1 ml of TE buffer (pH 8.0) was then added.

DNA Extraction

An equal volume of phenol/chloroform/isoamyl alcohol solution (5 ml) was added to the lysate, mixed by inversion, and centrifuged for 20 minutes at 27000 g. The upper (aqueous) layer containing the DNA was placed in a fresh centrifuge tube, and an equal volume of chloroform/isoamyl alcohol was added, mixed by inversion, and centrifuged for 15 minutes at 27000 g. The upper layer was again removed to a clean centrifuge tube a 1/10 volume of 3 M sodium acetate and two volumes of cold (-20°C) absolute ethanol were added, and the solution was then placed at -20°C overnight to precipitate the DNA. The solution was then centrifuged for 30 minutes at 27000 g and the supernatant discarded. The pellet was washed with 5 ml of 70% ethanol, dried in a desiccator for 30 minutes, and then resuspended in 250 µl of sterile TE buffer.

Restriction Enzyme digestion of Total DNA

10 µl of *S.aureus* DNA was added to an eppendorf tube containing 2 µl of 10x restriction enzyme buffer and 6 µl of distilled water. 2 µl of the restriction enzyme *Hin*dIII was then added and the mixture incubated at 37°c for 2 hours.

Preparation of a 1% Agarose gel

0.5 g of agarose was added to 50 ml of 1x TBB and boiled for 5 minutes in a round bottomed flask. The agarose was cooled to 50°C then poured onto a levelled perspex plate edged with adhesive tape. A comb was inserted into the agarose solution to create wells and the gel was allowed to solidify for 30 minutes. The comb was then extracted, the adhesive tape was removed, and the gel slab was placed in an electrophoresis tank. 1x TBB was added to the tank until the gel was submerged to a depth of 1-2 mm.

Agarose Gel Electrophoresis

10 µl of *S.aureus* DNA (either uncut, or digested with *Hin*dIII) was mixed with 5 µl of Bromophenol Blue Tracking dye and loaded in a well of a 1% agarose minigel. The gel was run at 80 volts for 90 minutes. The gel was then stained with ethidium bromide (0.5 µg/ml) for 30 minutes, and photographed using KodaK T-max film.

4.4 RESULTS

4.4.1 PHAGE TYPING WITH AND WITHOUT HEAT SHOCK

The phage types of the PN MRSA and the AMSSA strain were compared using the basic international set of 23 bacteriophage. For further comparison two high level methicillin resistant strains (MRSAT, WN MRSA), two low level methicillin resistant strains (MRSAB and MRSAG), and three methicillin sensitive strains (SA1, SA2, SA4) were also typed.

The raw results of the phage typing with and without heat shock are given in Table 4.2, pg 59. The samples are graded depending on the number of plagues produced.

±	1 - 19 plaques	= very weak reaction
+	20 - 49 plaques	= weak reaction
++	≥ 50 plaques	= strong reaction
SCL	semiconfluent	= strong reaction
CL	confluent	= strong reaction

When the final phage type of a strain is recorded only strong and weak reactions are counted. This is the 'phage pattern' and is listed for each isolate in Table 4.4.

Of the nine strains tested three were typable and six were nontypable (ie gave no reaction) using routine phage typing.

The three typable strains included the two high level methicillin resistant strains (MRSAT and WN MRSA) and a methicillin sensitive strain (SA2). All three differed in phage type. Of the six nontypable strains three were typable when cultures were heat shocked before typing. These strains included the two low level methicillin resistant isolates (MRSAB and MRSAG) and a methicillin sensitive strain (SA1). Again all three differed in their phage pattern after heat shock.

Of the six nontypable strains three were still nontypable even when cultures were heat shocked prior to typing. These strains included the PN MRSA (a 'high level' MRSA strain) and the methicillin sensitive strain (AMSSA) isolated later from the same patient. The other strain was a methicillin sensitive strain (SA4) isolated from a nasal swab of a healthy individual.

4.4.2 REVERSE PHAGE TYPING

In reverse phage typing bacteriophage from the strain to be typed are isolated and their lytic properties tested against a standard set of *S.aureus* bacteria. The results are given in Table 4.3. The same grading system was used as for phage typing. From this raw data the recorded reverse phage type of each organism is given in Table 4.4 (strong are weak reactions are included).

Of the nine strains tested two were nontypable, these included the low level methicillin resistant strain (MRSAG) and a methicillin sensitive strain (SA1). Of the seven typable strains none had identical reverse phage types. However the reverse phage types of the PN MRSA, the AMSSA strain and another high level methicillin resistant strain from Palmerston North Hospital (MRSAT) were similar, in that, the PN MRSA produced phage which lysed eight hosts, the AMSSA strain produced phage which lysed five of these hosts and no others, while the MRSAT strain produced phage which lysed six of these hosts and no others.

4.4.3 PLASMID PROFILES

To compare the plasmids of the PN MRSA and the AMSSA strain, total DNA was extracted and the plasmids separated on a 1% agarose gel run at 80 volts for 90 minutes.

Three other MRSA strains and three methicillin sensitive strains were included for comparison. The plasmid profiles of these *S.aureus* strains are shown in figure 4.1. The chromosomal DNA band is marked by the abbreviation (c/s). All the other bands represent plasmids.

Of the 8 strains examined two did not contain plasmids. These two were methicillin sensitive strains isolated from nasal swabs of healthy individuals. The other strains examined contained a variety of plasmids.

The PN MRSA contained two plasmids, a large plasmid which migrated in the gel just below the chromosomal band (plasmid 1 figure 4.1) and a small plasmid (plasmid 2 figure 4.1). The faint band seen just above plasmid 2 is the open circular

form of this plasmid.

The AMSSA strain also contained both plasmid 1 and plasmid 2. In addition the AMSSA strain contained a third plasmid (plasmid 3 figure 4.1) which migrated slightly lower in the gel than plasmid 1.

Two other MRSA strains (MRSAT and MRSAB), isolated from Palmerston North Hospital, also contained plasmid 1, however neither contained plasmid 2.

The other strains tested varied greatly in the number and type of plasmids they possessed.

4.4.4 DIGESTION OF TOTAL DNA WITH *HIN*DIII

Total DNA from the PN MRSA and the AMSSA strain was cleaved with the restriction enzyme *Hin*dIII. The results are shown in figure 4.2. The two strains produced similar banding patterns. Other strains were more heterogeneous in their banding patterns (patterns not shown).

4.5 DISCUSSION

The AMSSA strain was compared with the PN MRSA and various other methicillin resistant and sensitive *S.aureus* isolates using phage typing, reverse phage typing, plasmid profiles and restriction enzyme (*Hin*dIII) digestion of total DNA.

The PN MRSA and the AMSSA strain were both nontypable using routine phage typing even when cultures were heat shocked prior to typing. Their reverse phage types were also similar in that all the bacterial strains that the phage from the AMSSA strain could lyse, the phage from the PN MRSA could also lyse (although the PN MRSA's phage could lyse three additional hosts). None of the other strains examined shared such similarities when the combination of both phage type and reverse phage type were considered.

The PN MRSA and the AMSSA strain had similar plasmid profiles. Both contained plasmid 1 and plasmid 2. The AMSSA strain also contained an additional plasmid,

plasmid 3. Plasmid 2 was not found in any of the other isolates tested and indeed the other strains examined varied greatly in the number and type of plasmids they possessed.

The PN MRSA and the AMSSA strain had similar banding patterns when total DNA was digested with the restriction enzyme *Hin*dIII. However detailed comparison here was not possible because of the large number of fragments generated. This problem was overcome by using the restriction enzyme *Sma*I which recognises a sequence relatively rare in *S.aureus* DNA, this is discussed in the next chapter.

In conclusion, although differences could be seen between the PN MRSA and the AMSSA strain using all of the comparative methods discussed, in all instances, the two isolates were clearly more similar to each other than they were to any of the other methicillin resistant or sensitive isolates examined. This suggests a relationship between the AMSSA strain and the PN MRSA.

Table 4.2. Phage Patterns of *S.aureus* isolates

	PN MRSA		AMSSA		WN MRSA		MRSAT		MRSAB		MRSAG		SA1		SA2		SA4	
		HS		HS		HS		HS		HS		HS		HS		HS		HS
29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Cl	Cl	-	-
52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-
52A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
79	-	-	-	-	-	-	-	-	-	-	-	-	∓	++	-	-	-	-
80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	SCl	-	-
71	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
81	-	-	-	-	-	++	-	-	-	-	-	-	-	-	++	++	-	-
6	-	-	-	-	++	++	-	-	-	++	-	-	-	-	-	-	-	-
42E	-	-	-	-	-	++	-	-	-	-	-	-	-	∓	++	++	-	-
47	-	-	-	-	-	++	-	-	-	++	-	-	-	∓	-	++	-	-
53	-	-	-	-	-	++	-	-	-	++	-	-	-	-	∓	∓	-	-
54	-	-	-	-	++	++	-	-	-	++	-	-	-	++	++	++	-	-
75	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-	++	-	-
77	-	-	-	-	-	∓	-	-	-	++	-	++	-	-	SCl	SCl	-	-
83A	-	-	-	-	Cl	Cl	++	++	-	-	-	-	-	-	∓	∓	-	-
84	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	∓	-	-
85	-	-	-	-	∓	++	-	-	-	-	-	-	-	-	-	-	-	-
94	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
95	-	-	-	-	-	∓	-	-	-	-	-	++	-	-	-	-	-	-
96	-	-	-	-	-	-	-	-	-	-	-	-	-	∓	-	++	-	-

HS = After Heat Shock

Table 4.3.
Reverse Phage Patterns of *S.aureus* isolates

	85653	86203	PNMRSA	AMSSA	WNMRSA	MRSAT	MRSAB	MRSAG	SA1	SA2	SA3
PS6	++	++	++	±	SCl	-	±	-	-	-	-
PS42E	-	-	-	-	-	-	+	-	-	-	-
PS47	++	++	++	++	++	SCl	+	-	-	-	-
PS75	-	-	-	-	-	-	±	-	-	-	-
PS84	-	-	-	-	-	-	-	-	-	-	-
PS85	++	++	++	-	++	-	-	-	-	-	-
653	-	SCl	Cl	Cl	-	++	±	-	-	-	-
614	++	++	++	+	++	++	++	-	-	-	-
1653	+	±	-	±	+	-	-	-	-	-	-
1671	-	++	++	++	-	++	-	-	-	-	-
1691	-	-	-	-	-	-	-	±	-	-	-
W57	Cl	Cl	SCl	+	Cl	++	Cl	-	-	Cl	-
1030	++	++	+	-	++	++	Cl	-	-	-	++
18042	++	±	±	-	+	-	++	-	-	-	-

Table 4.4.
Phage Type and Reverse Phage Type of *S.aureus* isolates

	Phage Type		
Strains	Before Heat Shock	After Heat Shock	Reverse Phage Type
PN MRSA	Nontypable	Nontypable	6/47/85/653/614/1671/ W57/1030
AMSSA	Nontypable	Nontypable	47/653/614/1671/W57
WN MRSA	6/54/83A	81/6/42E/47/53/54/ 75/83A/85	6/47/85/614/1653/ W57/1030/18042
MRSAT	83A	83A	47/653/614/1671/ W57/1030
MRSAB	Nontypable	6/47/53/54/77/84	42E/47/614/ W57/1030/18042
MRSAG	Nontypable	77/95	Nontypable
SA1	Nontypable	79/54	Nontypable
SA2	29/81/42E/54/77	29/52/55/81/42E/47/54/ 75/77/96	W57
SA4	Nontypable	Nontypable	1030

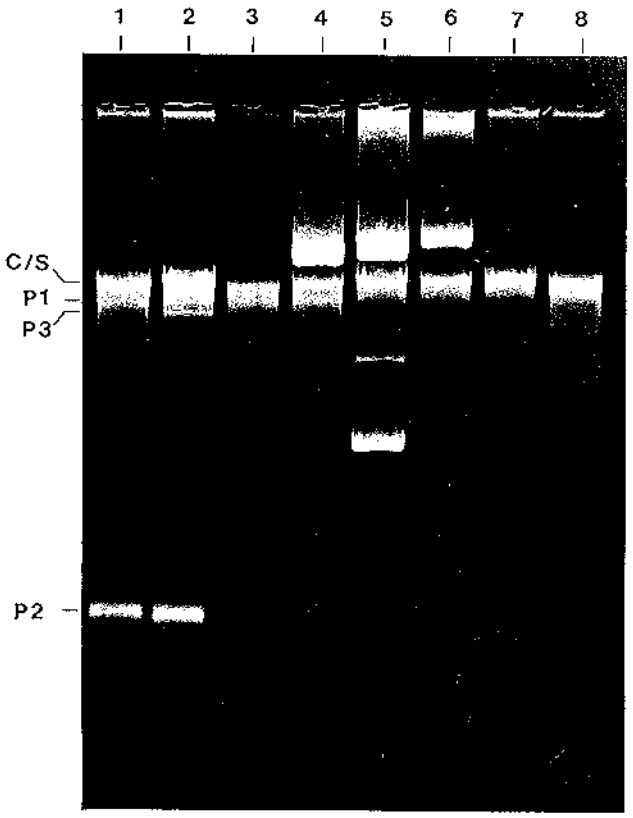


Figure 4.1. Plasmid profiles of *S.aureus* isolates. Lane 1. PN MRSA; Lane 2. AMSSA ; Lane 3. MRSAT; Lane 4. MRSAB; Lane 5. MRSAG; Lane 6. SA1; Lane 7. SA2; Lane 8. SA4.

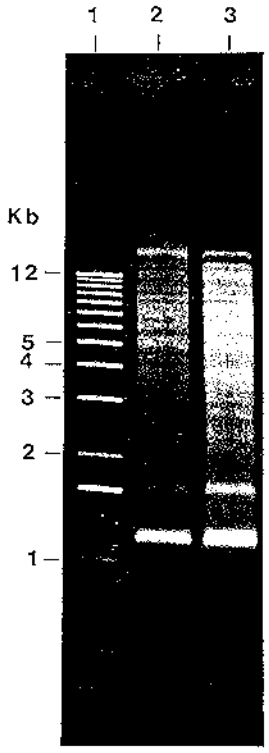


Figure 4.2. *Hin*dIII restriction patterns of *S.aureus* isolates. Lane 1. 1-kilobase ladder; Lane 2. PN MRSA; Lane 3. AMSSA strain.

CHAPTER 5
COMPARISON OF *S.AUREUS* ISOLATES USING *SMA*I RESTRICTION ENZYME ANALYSIS

5.1 INTRODUCTION

The restriction enzyme *Sma*I recognizes the sequence CCCGGG, this sequence is only rarely found in *S.aureus* DNA, so when the DNA is cleaved with *Sma*I only about 16 fragments, ranging in size from 10 to 500 kilobases, are generated. These fragments are too large to be separated by conventional agarose gel electrophoretic conditions but can be separated using pulsed-field gel electrophoresis (PFGE). The small number of bands generated is an advantage because it is easier to detect similarities or differences in the fragmentation patterns between strains, so this approach was used to further compare the PN MRSA with the AMSSA strain.

The DNA digests produced by *Sma*I were probed with the 1.1 kb PBP2a gene probe to identify the fragment or fragments, in the MRSA strains, which possessed the *mec* gene.

5.2 MATERIALS

Preparation of *S.aureus* DNA Plugs

Luria Glucose Broth plus Tris-HCl	
NaCl	4.0 g
Tryptone (Bacto)	8.0 g
Yeast Extract (Difco)	4.0 g
Glucose (0.2% W/V)	1.6 g
pH to 7-7.5 with 5 M NaOH	0.4 ml
1 M Tris-HCl (pH 7.5)	16.0 ml
Distilled water to	800.0 ml
TES Buffer	
1 M Tris-HCl (pH 7.8)	15.0 ml
5 M NaCl	5.0 ml
0.2 M EDTA	12.5 ml
Distilled water to	500.0 ml
2% Low Melting Point Agarose	
SeaKem agarose (HGT, FMC)	0.2 g
Sterile distilled water to	10.0 ml
Lysostaphin solution	
1 mg/ml Lysostaphin	200 µl
10% sodium lauryl sarcosine	200 µl
0.5 M EDTA to	2.0 ml
Pronase Solution	
20 mg/ml Pronase type XIV	200 µl
10% Na lauryl sarcosine	200 µl
0.5 M EDTA to	2.0 ml
TE Buffer	
1 M Tris-HCL (pH 7.5)	10.0 ml
0.2 M EDTA	5.0 ml
Distilled water to	1000.0 ml
100 mM Phenylmethane sulphonyl fluoride (PMSF)	
PMSF	0.017 g
isoproyl alcohol to	1.0 ml
store at -20°c	

Agarose gel electrophoresis

Tris-Borate Buffer (10x TBB)	
Trizma Base	108.8 g
EDTA (disodium salt)	9.3 g
Boric Acid	55.0 g
adjust pH to 8.2	
Distilled water to	1000.0 ml
0.5x TBB	
10x TBB	100.0 ml
Distilled water to	2000.0 ml
1.2% Agarose gel	
Agarose	1.2 g
0.5x TBB	100.0 ml
1.2% Low Melting Point Agarose	
SeaKem agarose (HGT, FMC)	0.12 g
0.5x TBB	10.0 ml

Southern Blotting of CHEF-DRII gels

0.4M NaOH	
NaOH	32.0 g
Distilled water to	2000.0 ml
0.5M Tris-HCL (pH 7.0)	
Trizma Base (Sigma)	60.55 g
HCL to pH 7.0	
Distilled water to	1000.0 ml
20x Standard Saline Citrate (SSC)	
NaCL	175.32 g
Sodium citrate	88.23 g
Distilled water to	1000.0 ml
2x SSC	
20x SSC	10.0 ml
Distilled water to	100.0 ml

5.3 METHODS

This method follows the Bio-Rad (P Schad US/EG Bulletin 1753 BioRad) method and the method used by Wada *et al* (1991), with a few modifications.

5.3.1 PREPARATION OF DNA PLUGS

Two to four *S.aureus* colonies, obtained from growth on a BHI plate, were inoculated into 5 ml of Luria Glucose broth plus Tris-HCl and incubated at 37°c overnight with shaking. 500 µl of the overnight culture was then centrifuged for 3 minutes in an eppendorf microfuge and the pellet washed once in 500 µl of TES buffer before resuspending in 500 µl of TES. The cell suspension was then placed at 50°c for 5 minutes before mixing with 500 µl of 2% low melting point agarose (SeaKem HGT, FMC) equilibrated to 50°c. 100 µl aliquots of the agarose-cell suspension were added to plug moulds and left to solidify for 15 minutes at 4°c. The agarose plugs were then pushed into a universal containing 2 ml of lysostaphin solution and incubated at 37°c for 48 hours. The lysostaphin solution was then removed and the plugs rinsed once in 5 ml of sterile water before being resuspended in 2 ml of pronase solution and incubated at 50°c for 24 hours. The plugs were then washed four times in 5ml of TE buffer for 1 hour at room temperature with gentle agitation. During the second wash 50 µl of 100 mM PMSF was added to inactivate any residual pronase. The plugs were then resuspended in 5ml of TE buffer and stored at 4°c until required.

5.3.2 DIGESTION OF *S.AUREUS* DNA WITH *SMA*I AND SEPARATION OF BANDS USING PULSED-FIELD GEL ELECTROPHORESIS

A 2 mm strip was cut off a DNA plug and placed in an eppendorf tube containing 300 µl of 1x restriction enzyme buffer and incubated for 1 hour at room temperature. The buffer was then removed and 300 µl of fresh 1x enzyme buffer was added. 2 µl of *Sma*I was added and the plug incubated overnight at 25°c. The following morning

another 2 µl of *Sma*I was added and the plug incubated at 25°c for 4 hours. The buffer was then removed and the plug washed in 500 µl of TE buffer for 30 minutes at room temperature. The plug was then added to 500 µl of 0.5x TBB and left for 15 minutes before being placed in a well of a 1.2% agarose gel. A lambda concatemer plug (Bio-Rad) 4 mm wide was also added to one of the wells as a size standard. The wells were then filled with 1.2% low melting point agarose and the gel left at room temperature for 15 minutes to allow the agarose to harden.

Two litres of 0.5x TBB were poured into the CHEF-DRII (Bio-Rad) electrophoresis chamber, and the water chiller and pump turned on to equilibrate the buffer to 8°c. The gel was then placed in the chamber so that the bottom corners were resting against the two gel stops. The flow rate on the pump was set to 45. The switching time on the Pulsewave 760 Switcher was set to 20 seconds with a run time of 20 hours and the voltage on the power supply set to 200 volts (6.0 V/cm).

5.3.3 SOUTHERN BLOTTING OF CHEF-DRII GELS

The CHEF-DRII gel was stained with ethidium bromide (0.5 µg/ml) for one hour with constant shaking. The gel was photographed using a UV light box and then irradiated for a further 60 seconds to ensure the DNA had been cleaved. The gel was then soaked in 0.4 M NaOH for 20 minutes. The DNA was then transferred onto a nylon membrane (Hybond-N+) using the method of Southern (1975). The wells of the blotting stand were filled with 0.4 M NaOH. Two sheets of Whatman 3MM paper (310 mm x 156 mm) soaked in 0.4 M NaOH were placed on the blotting stand and the air bubbles smoothed out. The stand was then covered with gladwrap and a grid, 2 mm smaller than the gel, was cut out in the middle of the stand. The gel was then placed, well side down, on top of the grid. A nylon membrane and two sheets of Whatman 3MM paper (2 mm larger than the gel), were presoaked in distilled water and placed on top of the gel. Two sheets of dry Whatman 3MM paper were then placed above the nylon membrane followed by a stack of dry paper towels and a heavy weight. DNA transfer was allowed to occur over a 24 to 36 hour period. The nylon membrane was then removed and soaked in 0.5 M Tris-HCl (pH 7) for 5

minutes. The membrane was then rinsed briefly in 2x SSC, blotted dry on Whatman 3MM paper, and baked in a 80°c oven for at least 2 hours. The membrane was then stored until required.

5.3.4 PROBING THE *SMA*I DIGESTS FOR THE *MEC* GENE

The 1.1 kb PBP2a gene probe (Archer and Pennel 1990) was labelled either with ^{32}P or a non-radioactive digoxigenin-dUTP label (see method earlier, section 3.3.5). Prehybridisation, hybridisation and washing conditions were also as described earlier.

5.4 RESULTS

DNA from the PN MRSA, the AMSSA strain, two other high level methicillin resistant strains (WN MRSA and MRSAT), two low level methicillin resistant strains (MRSAB and MRSAG) and three methicillin sensitive strains (SA1, SA2 and SA4) were cleaved with the restriction enzyme *Sma*I and the fragments separated using PFGE. The results are shown in figure 5.1a.

The separated fragments were probed with the 1.1 kb PBP2a gene probe. This was done in parallel using a ^{32}P labelled probe and a nonradioactive digoxigenin-dUTP probe. No bands were highlighted when the digoxigenin-dUTP label was used. However six bands were highlighted when the *mec* gene probe was labelled with ^{32}P (Figure 5.1b). This included a weak signal (probably due to *E.coli* vector DNA sequences in the probe) to the top (largest) band of the lambda concatemer ladder. Strong signals were produced from the hybridisation of the *mec* gene probe to a single fragment in each of the restriction profiles of five MRSA strains. The probe did not hybridise to any of the *Sma*I fragments from the AMSSA strain or to any of the methicillin sensitive isolates.

5.5 DISCUSSION

Discussion of these results is deferred to the general discussion.

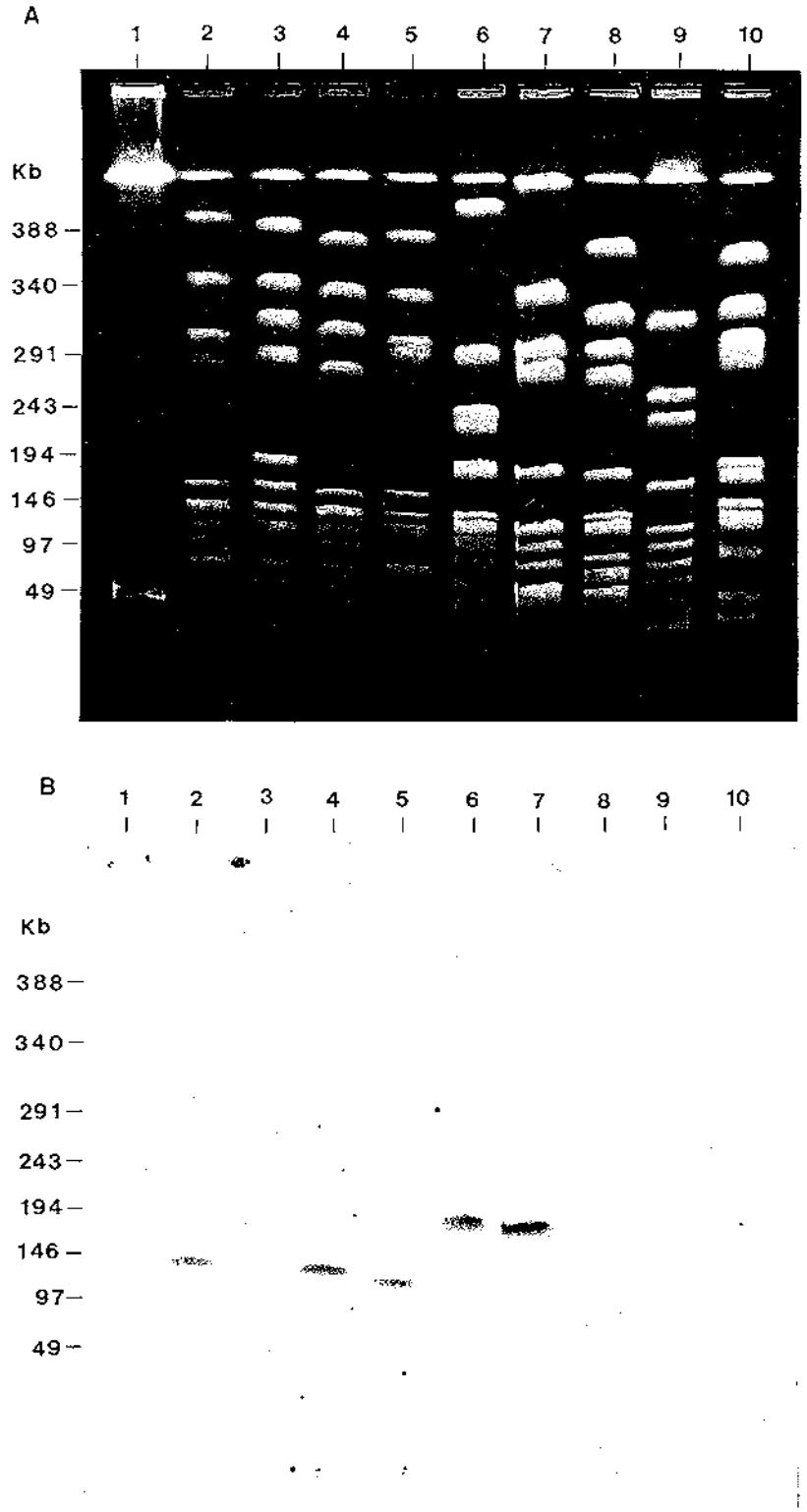


Figure 5.1. a) Pulsed field gel electrophoresis of *Sma*I digested *S.aureus* DNA and b) hybridisation with a ^{32}P-labelled *mec* gene probe. Lane 1. Lambda DNA concatemer size standard; Lane 2. PN MRSA; Lane 3. AMSSA; Lane 4. WN MRSA; Lane 5. MRSAT; Lane 6. MRSAB; Lane 7. MRSAG; Lane 8. SA1; Lane 9. SA2; Lane 10. SA4.

Chapter 6
GENERAL DISCUSSION

A methicillin sensitive strain of *S.aureus* (AMSSA) was isolated by Palmerston North Hospital from a patient who had been infected with the Palmerston North outbreak MRSA strain. The hospital was concerned that this AMSSA strain may have been derived *in vivo* from the PN MRSA by a reversible mutation, and if so, could rapidly revert to the resistant phenotype when exposed to methicillin. This thesis was largely concerned with a comparison of the PN MRSA and the AMSSA strain and addresses two questions:

1) Is it likely that the sensitive strain was derived *in vivo* from the resistant strain? and if so

2) is it likely that exposure of the sensitive strain to methicillin would lead to resistance ? (ie is sensitivity due to a reversible point mutation or small deletion?)

Since the second question was critical and urgent from a clinical standpoint it was addressed first. A logical approach to this question would be to expose the AMSSA strain and other methicillin sensitive isolates to increasing concentrations of methicillin and see whether the AMSSA strain develops resistance to high levels of methicillin more quickly than other methicillin sensitive strains. However Berger-Bachi *et al* (1989) have shown that *in vitro* selected resistance is different from natural methicillin resistance. Resistance in these isolates is not due to the acquisition of the PBP2a gene but rather due to chromosomal mutations which lead to a lowering in the affinity of preexisting PBPs to methicillin. Consequently if the AMSSA strain were exposed to methicillin *in vitro* the resistance which develops may not be due to reversion of the PBP2a gene to wild type, but rather due to mutations which alter preexisting PBPs. Hence these results may not necessarily parallel what would occur *in vivo*.

Consequently to achieve a more definite answer the AMSSA strain, was examined for the presence or absence of the PBP2a gene using a *mec* gene probe. If sensitivity in the AMSSA strain was due to either an easily reversible point mutation or to a

small deletion the isolate should contain sequences which would hybridise with the *mec* gene probe. However if the isolate had entirely 'lost' the *mec* gene it would not hybridise with the PBP2a gene probe.

Probing for the presence or absence of the *mec* gene

The majority of MRSA strains isolated in New Zealand in recent years are resistant to only low levels of methicillin (ie MICs 8 to 32 μg/ml), and unlike most of the MRSA found overseas, are not multiply resistant (CDNZ suppl.1 1992).
In multiply resistant MRSA strains the *mec* gene and many of the other resistant determinants are found on the chromosome as part of a large '*mec* gene complex' (up to 50 kilobases in size) (Inglis *et al* 1990, Dubin *et al* 1992). The low level singularly resistant MRSA found in New Zealand must lack that part of the *mec* gene complex that confers resistance to antibiotics other than methicillin and, as they are resistant to only low levels of methicillin it is possible that they may also lack the *mec* gene itself. Hence some other mechanism, besides the possession of a gene coding for PBP2a, may mediate the low level of methicillin resistance shown by these isolates. For this reason these isolates were also probed for the presence or absence of the *mec* gene.

Both the low level singularly resistant MRSA strains (MIC 8-32 g/ml) and the high level multiply resistant MRSA strains (MIC ≥64μg/ml) hybridised with the *mec* gene fragment (Figure 3.1). None of the methicillin sensitive isolates MIC ≤4μg/ml hybridised with the *mec* gene probe. Therefore there was total correlation between the MIC at which a strain is considered resistant (≥8 μg/ml) and possession of the *mec* gene.
The AMSSA strain (MIC = 4 μg/ml) did not hybridise with the *mec* gene probe.
The 1.1 kilobase PBP2a gene probe covers most of the structural sequence for the PBP2a gene. Therefore if the AMSSA strain was derived *in vivo* from the PN MRSA its sensitivity was not due to an easily reversible point mutation or to a small deletion, but rather due to the deletion of at least 1.1 kilobases which covers most

of the coding region for PBP2a. It follows from this that the AMSSA strain is no more likely than any other methicillin sensitive isolate to develop resistance to methicillin following exposure to the drug.

As all the low level methicillin resistant isolates examined hybridised with the *mec* gene probe, this implies that the *mec* gene and not some other mechanism is responsible for methicillin resistance. The reason why these isolates express only low levels of resistance was not investigated further. However other workers have shown that the level of resistance does not correlate with the amount of PBP2a present (Chambers 1988). It is known that additional chromosomally-located genes not linked to *mec* are essential for the expression of methicillin resistance (Berger-Bachi and Kohler 1983, Kornblum *et al* 1986). These genes termed *fem* (factors essential for the expression of methicillin resistance) are found in both methicillin resistant and susceptible strains. Two of these factors *fem*A and *fem*B are involved in the peptiglycine cross-bridge formation of *S.aureus* peptidoglycan (Berger-Bachi 1992). Inactivation of these genes by Tn*551* insertion lowers the level of methicillin resistance. Perhaps one or all of these *fem* genes are absent or mutated in low level MRSA strains.

The reason why these low level MRSA isolates were not multiply resistant was again not investigated. However these isolates may lack the appropriate transposon target sites or insertion sequences, such as IS*257*, surrounding the *mec* gene which are thought to promote the accumulation of resistance genes in this area of the chromosome. This merits further investigation.

Was the AMSSA strain derived *in vivo* from the PN MRSA?

As the AMSSA strain does not possess the *mec* gene it is unlikely that it will rapidly develop resistance to methicillin following exposure to the drug. However, the question as to whether this methicillin sensitive strain was derived *in vivo* from the PN MRSA still needs to be answered.

Methicillin resistant strains have been converted to sensitivity *in vitro* and in all cases

the methicillin sensitive derivatives had lost the entire *mec* gene and all or part of the *mec* gene complex. Whether or not deletions of a similar size occur *in vivo* is not known.

Inglis *et al* (1993) have recently reported evidence which suggests that the deletion of large amounts of DNA may occur from 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 of isolation was not known in two of the three cases. Therefore the difference seen may have been due to an insertion rather than a deletion of DNA.

Consequently to address the above question, the AMSSA strain was compared with the PN MRSA and other methicillin resistant and sensitive *S.aureus* strains using a variety of techniques.

The comparison included the following:

1) Colony Morphology
2) Growth Rate
3) Phage Typing
4) Reverse Phage Typing
5) Plasmid Profiles
6) *Hin*dIII digests
7) *Sma*I digests

Colony Morphology

The recognition of MRSA strains must depend on tests to identify antibiotic resistance but nevertheless a reasonably reliable indicator of the presence of such strains is the observation that they produce colonies smaller than typical methicillin sensitive *S.aureus* isolates.

The AMSSA strain is unusual in that although it is methicillin sensitive it produces colonies similar in size and appearance to methicillin resistant strains (Figure 2.2 and 2.3). This may suggest a relationship between the AMSSA strain and methicillin

resistant strains.

Growth Rate

All the methicillin resistant and methicillin sensitive *S.aureus* strains examined had similar growth rates in BHI broth Graph 2.1. The reason for the lack of correlation between growth rate in broth and colony size on agar was not investigated. However these results imply that any extrapolation from colony size on agar to the growth rate *in vivo* may be invalid because the *in vivo* environment may be more similar to a broth rather than an agar culture.

Phage Type and Reverse Phage Type

S.aureus isolates are phage typed according to their sensitivity to an internationally standardised set of 23 bacteriophage. As the phage all absorb to the same receptor located in the peptidoglycan-teichoic acid complex, differences in susceptibility are related to postadsorption phenomena including restriction-modification systems and host, plasmid, or phage-mediated blockage of phage development (Davis et al 1990). The PN MRSA and the AMSSA strain were both nontypable using routine phage typing even when cultures were heat shocked prior to typing (Table 4.4), this often increases an isolates susceptibility to phage. The other isolates varied in the number and type of phage they were susceptible to.

If an isolate cannot be typed by phage typing it can often be typed by reverse phage typing. Reverse phage typing involves evaluating the lytic activities of phage from the test strain against a set of standard indicator strains.

The reverse phage types of the PN MRSA and the AMSSA strain were similar in that all the bacterial strains that the phage from the AMSSA strain could lyse the phage from the PN MRSA could also lyse. Although the PN MRSA's phage could lyse three additional hosts. Interestingly, two other high level methicillin resistant strains (WN MRSA and MRSAT) also had reverse phage types similar to those of

the PN MRSA and the AMSSA strain. However when the combination of both phage type and reverse type were considered the PN MRSA and the AMSSA strain were more similar to each other than they were to any of the other isolates examined.

Plasmid Profiles

Both isolates had similar plasmid profiles (Figure 4.1). The PN MRSA and the AMSSA strain were the only two isolates tested which contained both plasmid 1 and plasmid 2. The AMSSA strain also contained an additional plasmid, Plasmid 3. The other strains tested varied considerably in the number and type of plasmids they possessed.

Digestion of total DNA with *Hin*dIII

The PN MRSA and the AMSSA strain had similar banding patterns when total DNA was cleaved with the restriction enzyme *Hin*dIII (Figure 4.2). However detailed comparison here was not possible because of the large number of fragments generated. This problem was overcome by using a restriction enzyme, *Sma*I.

Digestion of total DNA with *Sma*I

*Sma*I recognises a sequence which is relatively rare in *S.aureus* DNA so the chromosome is cleaved into a relatively small number of fragments. The fragments while too large to be separated by conventional agarose gel electrophoresis were separated by pulsed field gel electrophoresis (PFGE).

All the strains examined produced different banding patterns when their DNA was cleaved with *Sma*I (Figure 5.1). However the banding patterns produced by the first four isolates, namely the PN MRSA, the AMSSA strain, and two 'high level multiply resistant' MRSA strains (WN MRSA and MRSAT) were more similar than the

banding patterns produced by the second five isolates which included two 'low level' MRSA strains and three methicillin sensitive strains. These results suggest that the AMSSA strain is more closely related to the PN MRSA and other high level multiply resistant MRSA strains than it is to other methicillin sensitive or low level methicillin resistant isolates.

Furthermore, the differences seen between the PN MRSA and the AMSSA strain when their DNA was cleaved with *Sma*I involved four DNA fragments, three of these, located in the 100-200 kilobase region of the gel, are consistent with the suggestion that the AMSSA strain was derived from the PN MRSA by a deletion, as the AMSSA strain had:

1)'lost' a 143 kb fragment,

2)'lost' a 104 kb fragment, and

3)'gained' a 203 kb fragment.

The loss of two fragments followed by the gain of a fragment may be explained by a deletion which spans a *Sma*I site (Figure 6.1), and in this case may represent a deletion of 44 kilobases of DNA from the AMSSA strain.

The deletion pattern proposed here is almost identical to the deletion pattern produced when a methicillin resistant strain ANS46 was converted to sensitivity *in vitro* by acriflavin treatment (Inglis et al 1990). The derived methicillin sensitive strain (ANS62) had:

1) lost a 145 kb fragment (which contained the *mec* gene),

2) lost a 110 kb fragment, and

3) gained a 215 kb fragment,

indicating a deletion of 40 kilobases of DNA had occurred from the *mec* region.

Equivalent differences were also seen between ANS46 and a methicillin sensitive strain isolated within months of ANS46, at the same hospital, but from a different patient (Inglis *et al* 1993). However in this case, the sequence of isolation was not known, so the differences seen may have been due to an insertion rather than a deletion of DNA.

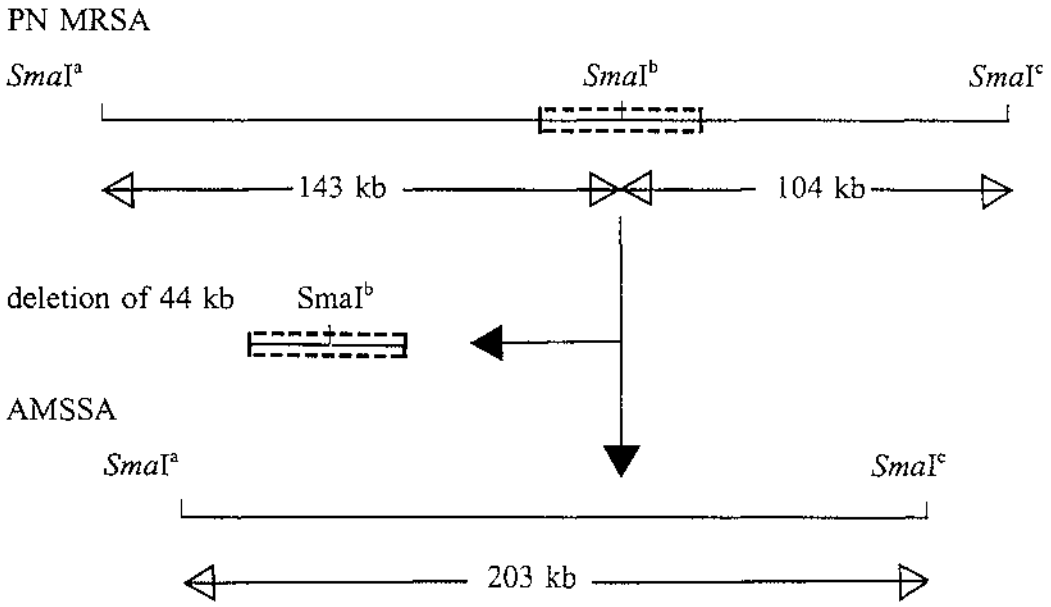


Figure 6.1. The AMSSA strain differs from the PN MRSA by the loss of two fragments and the gain of a fragment, this may be due to a deletion which spans a *Sma*I site.

When the *Sma*I fragments were probed for the presence or absence of the *mec* gene, as expected, all five methicillin resistant strains hybridised with the PBP2a gene fragment. None of the methicillin sensitive isolates, including the AMSSA strain, hybridised with the probe. This confirms earlier slot-blot results. In the PN MRSA strain the 143 kb fragment contained the *mec* gene. This fragment was missing from the restriction profile of the AMSSA strain. This is consistent with the deletion pattern proposed above, and equates with the *in vitro* results.

In Summary

Although differences could be seen between the PN MRSA and the AMSSA strain using all of the comparative methods discussed (ie phage typing, reverse phage typing, plasmid profiles, and genomic digests), in all instances the two isolates were clearly more similar to each other than they were to any of the other methicillin resistant or sensitive isolates examined. This suggests a relationship between the AMSSA strain and the PN MRSA. Furthermore, the differences seen between the two isolates when their DNA was cleaved with *Sma*I may be explained by a deletion which spans a *Sma*I site.

Therefore it seems plausible to assume that the AMSSA strain was in fact derived *in vivo* from the PN MRSA strain by a deletion of 44 kilobases of DNA from the *mec* region. This is the first reported instance of a deletion being found in sequential isolates from the same patient.

The *mec* gene complex (ie the *mec* and its surrounding resistance genes) has been estimated to span approximately 40 to 50 kilobases of DNA (Inglis *et al* 1990, Dubin *et al* 1992). This is equivalent to the size of the deletion proposed. Therefore the deletion probably spans not only the *mec* gene but the entire *mec* gene complex as well. This is consistent with earlier results which showed that the AMSSA had lost not only its resistance to methicillin but also its resistance to gentamicin as well (Table 3.1).

These results suggest that the *mec* region is unstable, and that under the appropriate conditions such as during physiological stress or in the absence of antibiotic selective agents, the *mec* gene and its surrounding resistant determinants may be lost from the chromosome of MRSA strains *in vivo*. How often this occurs is not known, however, Inglis and coworkers (1993) have reported two other instances where the *mec* region may have been lost from the chromosome of MRSA strains in the clinical environment.

The question remains as to what causes these MRSA strains to revert to sensitivity *in vivo*? Is the *mec* region so unstable that it is lost in the mere absence of antibiotic selective agents? Can other conditions, besides the absence of antibiotics, also bring about this loss? The answers to these questions could have important implications for the treatment of MRSA infections in the future and need to be investigated further.

LITERATURE CITED

Archer, G.L., and E. Pennell. (1990). Detection of methicillin resistance in staphylococci by using a DNA probe. Antimicrobial Agents and Chemotherapy. **34**: 1720-1724.

Barber, M. and M. Rozwadowska-Dowzenko. (1948). Infection by penicillin-resistant staphylococci. Lancet. **ii**: 641-644.

Barberis-Maino, L., B. Berger-Bachi, H. Webwe, W.D. Beck, and F.H. Kayser. (1987). IS*437*, a staphylococcal insertion sequence-like element related to IS*26* from *Proteus vulgaris*. Gene. **59**: 107-113.

Berger-Bachi, B. (1983). Insertional inactivation of staphylococcal methicillin resistance by Tn*551*. Journal of Bacteriology. **154**: 479-487.

Berger-Bachi, B. (1989). Genetics of methicillin resistance in *Staphylococcus aureus*. Journal of Antimicrobial Chemotherapy. **23**: 671-680.

Berger-Bachi, B., L. Barberis-Maino, A. Strassle, and F. Kayser. (1989). *Fem*A, a host-mediated factor essential for methicillin resistance in *Staphylococcus aureus*: Molecular cloning and characterization. Mol Gen Genet **219**: 263-269.

Berger-Bachi, B., and M.L. Kohler. (1983). A novel site on the chromosome of *Staphylococcus aureus* influencing the level of methicillin resistance: genetic mapping. FEMS Microbiological Letters. **20**: 305-309.

Berger-Bachi, B., A. Strassle, J.E. Gustafson, and F.H. Kayser. (1992). Mapping and characterization of multiple chromosomal factors involved in methicillin resistance in *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy. **36**: 1367-1373.

Berger-Bachi, B., A. Strassle, and F.H. Kayser. (1989). Natural methicillin resistance in comparison with that selected by in-vitro drug exposure in *Staphylococcus aureus*. Journal of Antimicrobial Chemotherapy. **23**: 179-188.

Beck, W.D., B. Berger-Bachi, and F.H. Kayser. (1986). Additional DNA in methicillin-resistant *Staphylococcus aureus* and molecular cloning of *mec* specific DNA. Journal of Bacteriology. **165**: 373-378.

Blair, J.E, and R.E.O Williams. (1961). Phage typing of staphylococci. Bull. Wld. Hlth. Org. **24**: 771-784

Blanchard, T.J., S.M. Poston, and P.J. Reynolds. (1986). Recipient characteristics in the transduction of methicillin resistance in *Staphylococcus epidermidis*. Antimicrobial Agents and Chemotherapy. **29**: 539-541.

Brett, M.S.Y, H. Heffernan, and S.G. Bowers. (1993). Evaluation of laboratory methods for the determination of methicillin susceptibility in New Zealand isolates of *Staphylococcus aureus*. New Zealand Journal of Medical Laboratory Science. **47**: 7-11.

Brown, D.F.J., and P.E. Reynolds. (1980). Intrinsic resistance to beta-lactam antibiotics in *Staphylococcus aureus*. FEBS letters. **122**: 275-278.

Casewell, M.W. (1986). Epidemiology and control of the 'modern' methicillin-resistant *Staphylococcus aureus*. Journal of Hospital Infection. 7: (Supplement A) 1-11.

CDNZ Supplement 1. (1992). Guidelines for the control of methicillin-resistant *Staphylococcus aureus* in New Zealand. p1-18.

Chambers, H.F. (1987). Coagulase-negative staphylococci resistant to ß-lactam antibiotics *in vivo* produce penicillin-binding protein 2a. Antimicrobial Agents and Chemotherapy. **31**: 1919-1924.

Chambers, H.F. (1988). Methicillin resistant staphylococci. Clinical Microbiology Reviews. **1**: 173-186.

Chikramane, S.G., P.R. Matthews, W.C. Noble, P.R. Stewart, and D.T. Dubin. (1991). Tn*554* inserts in methicillin-resistant *Staphylococcus aureus* from Australia and England: comparison with an American methicillin-resistant group. Journal of General Microbiology. **137**: 1303-1311.

Cookson, B., and I. Phillips. (1990). Methicillin-resistant staphylococci. Journal of Applied Bacteriology Symposium Supplement. 55S-70S.

Davies, H.G., and D.R. Martin. (1987). Heat Shocking as an adjunct to routine phage typing of *Staphylococcus aureus*. Journal of Hospital Infection. **10**: 4-9.

Dubin, D.T., S.G. Chikramane, B. Inglis, P.R. Matthews, and P.R. Stewart. (1992). Physical mapping of the *mec* region of an Australian methicillin-resistant *Staphylococcus aureus* lineage and a closely related American strain. Journal of General Microbiology. **138**: 169-180.

Dubin, D.T., P.R. Matthews, S.G. Chikramane, and P.R. Stewart. (1991). Physical mapping of the *mec* region of an American methicillin-resistant *Staphylococcus aureus* Strain. Antimicrobial Agents and Chemotherapy. **35**: 1661-1665.

Georgopapadakou, N.H., B.A. Dix and Y.R. Mauriz. (1986). Possible physiological functions of penicillin-binding proteins in *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy. **29**: 333-336.

Hacbarth, C.J., and H.F. Chambers. (1989a). Methicillin-resistant staphylococci: Genetics and mechanisms of Resistance. Antimicrobial Agents and Chemotherapy. **33**: 991-994.

Hacbarth, C.J., and H.F. Chambers. (1989b). Methicillin-resistant staphylococci: Detection methods and treatment of infections. Antimicrobial Agents and Chemotherapy. **33**: 995-999.

Heffernan, H., J. Stehr-Green, H. Davies, M. Brett, and S.Bowers. (1993). Methicillin resistant *Staphylococcus aureus* (MRSA) in New Zealand 1988-90. New Zealand Medical Journal. **106** 72-74.

Henze, U., T. Sidow, J. Wecke, H. Labischinski, and B. Berger-Bachi. (1993). Influence of *fem*B on methicillin resistance and peptidoglycan metabolism in *Staphylococcus aureus*. Journal of Bacteriology. **175**: 1612-1620.

Humble M.W. (1976). Imported methicillin-resistant *Staphylococcus aureus* infection: a case report. New Zealand Medical Journal. **84**: 476-478.

Inglis, B., P.R. Matthews, and P.R. Stewart. (1988). The expression in *Staphylococcus aureus* of cloned DNA encoding methicillin resistance. Journal of General Microbiology. **134**: 1465-1469.

Inglis, B., P.R. Matthews, and P.R. Stewart. (1990). Induced deletions within a cluster of resistance genes in the *mec* region of the chromosome of *Staphylococcus aureus*. Journal of General Microbiology. **136**: 2231-2239.

Inglis, B., W. El-Adhami, and P.R. Stewart. (1993) Methicillin-sensitive and -resistant homologues of Staphylococcus aureus occur together among clinical isolates. The Journal of Infectious Diseases. **167**: 323-328.

Jevons, M.P. (1961). "Celbenin"-resistant staphylococci. British Medical Journal. **1**, 124-125.

Jones, M. (1987). An outbreak of methicillin-resistant *Staphylococcus aureus* in Wellington Hospital. New Zealand Medical Journal of Laboratory Technology. 123-124.

Jones, M.R. (1987). Outbreak of methicillin-resistant *Staphylococcus aureus* infection in a New Zealand Hospital. The New Zealand Medical Journal. **100**: 369-373.

Kornblum, J., B.J. Hartman, R.P. Novick, and A. Tomasz. (1986). Conversion of a homogeneously methicillin-resistant strain of *Staphylococcus aureus* to heterogeneous resistance by Tn*551*-mediated insertional inactivation. European Journal of Clinical Microbiology. **5**: 714-718.

Kuhl, S.A., P.A. Pattee, and J.N. Baldwin. (1978). Chromosomal map location of the methicillin resistance determinant in *Staphylococcus aureus*. Journal of Bacteriology. **135**: 460-465.

Lyon, B.R., and R. Skurray. (1987). Antimicrobial resistance of *Staphylococcus aureus*: Genetic basis. Microbiological Reviews. **51**: 88-134.

Martin, D. (1987). Methicillin-resistant *Staphylococcus aureus* in New Zealand. New Zealand Medical Journal of Laboratory Technology. 121-122.

Martin, D.R., H.M Heffernan, and H.G Davies. (1989). Methicillin-resistant *Staphylococcus aureus*: an increasing threat in New Zealand hospitals. The New Zealand Medical Journal. **102**: 367-369.

Matsuhashi, M., M.D. Song, F. Ishino, M. Doi, M. Inoue, K. Ubukata, N. Yamashita, and M. Konno. (1986). Molecular cloning of the gene of a penicillin-binding protein supposed to cause high resistance to B-lactam antibiotics in *Staphylococcus aureus*. Journal of Bacteriology. **167**: 975-980.

Matthews, P.R., K.C. Reed, and P.R. Stewart. (1987). The Cloning of chromosomal DNA associated with methicillin and other resistances in *Staphylococcus aureus*. Journal of General Microbiology. **133**: 1919-1929.

Murakami, K., and A. Tomaz. (1989). Involvement of multiple genetic determinants in high-level methicillin resistance in Staphylococcus aureus. Journal of Bacteriology. **171**: 874-879.

National Committee for Clinical Laboratory Standards. (1990) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. 2nd ed. Villanova: NCCLS document; M7-A2

Novick, R.P. (1990). Staphylococci, p539-550 *In* B.D Davis, R. Dulbecco, H.N. Eisen and H.S Ginsberg (ed.), Microbiology 4th ed. J.B Lippincott Company. Philadelphia.

Parker, M.T. (1972). Phage-typing of *Staphylococcus aureus*. In Norris, J.R., D.W. Ribbons (Eds) Methods in Microbiology, London Academic Press, p1-28.

Reynolds, P.E. (1988). The essential nature of staphylococcal penicillin-binding proteins, p 243-351. *In* P. Actor, L. Daneo-Moore, M.L. Higgins, M.R.J. Salton, and G.D. Shockman (ed) Antibiotic inhibition of bacterial cell surface assembly and function. American Society for Microbiology, Washington, D.C.

Schad, P.A. (1992). Preparation and restriction digestion of *E.coli* chromosomal DNA in agarose plugs for use in PFGE. BioRad PFG Application #6. US/EG Bulletin 1753.

Scrimgeour, G. (1987) An outbreak of methicillin-resistant *Staphylococcus aureus* infection at Palmerston North Hospital. New Zealand Medical Journal of Laboratory Technology. 122-123.

Sjostrom, J.E., S. Lofdahl, and L. Phillipson. (1975). Transformation reveals a chromosomal locus of gene(s) for methicillin resistance in *Staphylococcus aureus*. Journal of Bacteriology. **123**: 905-915.

Skinner, S., B. Inglis, P.R. Matthews, and P.R. Stewart. (1988). Mercury and tetracyline resistance genes and flanking repeats associated with methicillin resistance on the chromosome of *Staphylococcus aureus*. Molecular Microbiology. **2**: 289-297.

Skurray, R.A., D.A Rouch, B.R Lyon, M.T Gillespie, J.M Tennent, M.E Byrne, L.J. Messerotti and J.W. May. (1988) Multiresistant *Staphylococcus aureus*: genetics and evolution of epidemic australian strains. Journal of Antimicrobial Chemotherapy. **21**: 19-38.

Song, M.D., S. Maesaki, M. Wachi, T. Takahashi, M. Doi, F. Ishino, Y. Maeda, K. Okonogi, A.Imada, and M.Matsuhashi. (1988). Primary structure and origin of the gene encoding the B-lactam-inducible penicillin-binding protein responsible for methicillin resistance in *Staphylococcus aureus*, p. 352-359. *In* P. Actor *et al* (ed.), Antibiotic inhibition of bacterial cell surface assembly and function. American Society for Microbiology, Washington, D.C.

Song, M.D., M. Wachi, M. Doi, F. Ishino, and Matsuhashi. (1987). Evolution of an inducible penicillin-target protein in methicillin-resistant *Staphylococcus aureus* by gene fusion. FEBS Letters. **221**: 167-171.

Stewart, G.C., and E.D. Rosenblum. (1980). Transduction of methicillin resistance in *Staphylococcus aureus*: recipient effectiveness and beta-lactamase production. Antimicrobial Agents and Chemotherapy. **18**: 424-432.

Tesch, W., A. Strassle, B. Berger-Bachi, D. O'Hara, P. Reynolds, and F.H. Kayser. (1988). Cloning and expression of methicillin resistance from *Staphylococcus carnosus*. Antimicrobial Agents and Chemotherapy. **32**: 1494-1499.

Thornsberry, C. (1988). The development of antimicrobial resistance in staphylococci. Journal of Antimicrobial Chemotherapy. **21**: supplement C, 9-16

Tomaz, A., S. Nachman, and H. Leaf. (1991). Stable classes of phenotypic expression in methicillin-resistant clinical isolates of Staphylococci. Antimicrobial Agents and Chemotherapy. **35**: 124-129.

Trees, D.L., and J.I. Iandolo. (1988). Identification of a *Staphylococcus aureus* transposon (Tn*4291*) that carries the methicillin resistance gene(s). Journal of Bacteriology. **170**: 149-154.

Ubukata, K., N. Yamashita, and M. Konno. (1985) Occurrence of a ß-lactam-inducible penicillin-binding protein in methicillin-resistant staphylococci. Antimicrobial Agents and Chemotherapy. **27**: 851-857.

Wada, A., Y. Katayama, K. Hiramatsu and T. Yokota. (1991). Southern hybridisation analysis of the *mec*A deletion from methicillin-resistant *Staphylococcus aureus*. Biochemical and Biophysical Research Communications. **176**: 1319-1325.

Waxman, D.J., J.L Strominger. (1983). Penicillin-binding proteins and the mechanism of action of beta-lactam antibiotics. Annu. Rev. Biochem. **52**: 825-869.

Wu, C.Y.E., J. Hoskins, L.C. Blaszczak, D.A Preston, and P.L. Skatrud. (1992). Construction of a water-soluble form of penicillin-binding protein 2a from a methicillin-resistant *Staphylococcus aureus* isolate. Antimicrobial Agents and Chemotherapy. **36**: 533-539.

Wyke, A.W. (1984) Isolation of five penicillin-binding proteins from *Staphylococcus aureus*. FEMS Microbiological Letters **22**: 133-138.