Genetic characterisation of resistance to metal ions in methicillin-resistant *Staphylococcus aureus*: elimination of resistance to cadmium, mercury and tetracycline with loss of methicillin resistance

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Summary. Susceptibility to six metal ions—cadmium (Cd), mercury (Hg), arsenate (Asa), arsenite (Asi), antimony (Sb) and zinc (Zn)—was tested in 23 independent isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) obtained from Guy's Hospital (GH) during 1984–1986, which included 10 isolates of the UK epidemic EMRSA-1 strain. Strains were also tested for resistance to antibiotics and the nucleic-acid-binding compounds propamidine isethionate and ethidium bromide. A further 19 methicillin-resistant isolates, including 10 EMRSA-1 were obtained from other sources. Ten methicillin-sensitive, antibiotic sensitive isolates were from Guy's Hospital. Resistance to Hg was associated with methicillin resistance in 19 of the 20 EMRSA-1 isolates, all of which were resistant to Cd. Resistance to Cd and Hg was found in 13 out of 22 other MRSA isolates. Hg resistance was not present in the methicillin-sensitive isolates which were often (13 out of 19) moderately resistant to Cd. Multiple resistance to metal ions, including resistance to Hg, Asa, Asi and Sb, was uncommon. Resistance to Cd (MIC > 32 mg/L or 8–16 mg/L) was associated with increased resistance to Zn. In 11 of the consecutive MRSA isolates from Guy's Hospital seven distinct strains were recognised by phage type. Methicillin resistance in these strains varied from 16 to 1024 mg/L at 30°C with a 2–8-fold lower minimum inhibitory concentration at 37°C indicating some degree of heterogeneity. Representatives of the EMRSA-1 strain had the higher levels of resistance. Loss of methicillin resistance occurred in 0.2–5.0% of colonies tested after storage at room temperature in 10 of these isolates. In some EMRSA-1 strains this loss was associated with loss of Cd, Zn and, usually, Hg resistance, and a 4-fold reduction in resistance to tetracycline. These resistance determinants were not transferred by transduction of plasmids from these strains and plasmid-free strains retained these markers, indicating that a chromosomal region is involved. These findings support evidence for a close relationship between the EMRSA-1 strain in the UK and the recent epidemic MRSA strain in eastern Australia. In the strains which lost methicillin resistance independently of other markers, Cd resistance and β -lactamase production were plasmid encoded, as shown by co-elimination and co-transduction of these markers. In addition to Cd and penicillin resistance, the markers encoded by these plasmids included resistance to Asa, Asi and Sb in two strains, resistance to Hg, propamidine isethionate and ethidium bromide in a further two isolates, and for resistance to Hg, Asa, Asi and Sb, kanamycin, neomycin and streptomycin, and erythromycin and clindamycin in one strain. Methicillin resistance was transduced from strain GH34 to strain 80CR5 with phage 80. MICs of methicillin (1024 mg/L) were higher for transductants than the donor strain (32–64 mg/L) at 30°C and showed little heterogeneity when tested at 37°C.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains were reported in the UK by Jevons as early as 1961.¹ The expression of resistance is often heterogeneous, with only a minority of cells (1 in 10^3 – 10^6) showing resistance to higher levels of methicillin.² In

heterogeneous strains, expression of resistance is greater at 30°C than at 37°C. Resistance is also affected by other growth conditions.³ Since 1970, strains responsible for outbreaks of infection have become dominant in several countries. Epidemic strains from eastern Australia and London are similar in characteristics, including the carriage of a large plasmid (c. 30 kb) coding for resistance to the nucleic-acid-binding (NAB) compounds propamidine isethionate and ethidium bromide.

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thionate (Pi) and ethidium bromide (Eb), to gentamicin, tobramycin and kanamycin and sometimes to penicillin (β -lactamase production) and trimethoprim.^{4,5} Plasmids coding for resistance to Pi, Eb, cadmium (Cd), mercury (Hg) and penicillin, occur in epidemic MRSA in south-east Asia,⁶ are found in Dublin⁷ and, occasionally, in the UK.^{4,8} The large (c. 30 kb) β -lactamase plasmids in hospital isolates of *S. aureus* isolated before 1970 carried different resistance determinants, often coding for multiple resistance to metal ions including Cd, Hg and arsenate (Asa).⁹⁻¹² Hg resistance was considered to be a useful marker for hospital strains.¹³ Many of these plasmids were related and belonged to the same incompatibility group (Inc I). Townsend *et al.*¹⁴ showed that a NAB plasmid from recent MRSA isolates in Australia also belonged to Inc I, which could have displaced the β -lactamase, metal-ion-resistance plasmid of the same incompatibility group. The genetic basis of a number of these plasmid types has been reviewed recently.¹⁵

In a recent study of hospital isolates of *S. aureus* in the UK, resistance to Hg was common only in methicillin-resistant isolates.¹⁶ In a large scale survey of *S. aureus* isolates in Denmark between 1967 and 1974,¹⁷ a decrease in the incidence of resistance to Hg was shown. Isolates of MRSA are frequently antibiotic multi-resistant and chromosomal resistance to erythromycin is common.^{18,19} Cd and Hg resistance is also chromosomally mediated in recent isolates of MRSA.²⁰ An epidemic strain predominating in London and the south-east of England has a characteristic phage type and antibiotic-susceptibility pattern and has been designated EMRSA-1.^{19,21} Loss of methicillin resistance is readily detected after storage at room temperature.²² Simultaneous loss of Cd and Hg resistance may occur with loss of methicillin resistance in *S. epidermidis*.²³ In *S. aureus*, Matthews *et al.*²⁴ showed that a large deletion of 30–40 kb, in an epidemic strain from eastern Australia, contained sequences in a 24–27-kb fragment responsible for resistance to methicillin, Hg and Cd and for resistance to tetracycline.^{24,25} Additional chromosomally determined resistance to tetracycline and to minocycline also occurs in Australian MRSA isolates²⁶ and in EMRSA-1 isolates in the UK.¹⁹

Methicillin resistance is often difficult to transduce in *S. aureus*. The recovery of transductants has been shown to depend on the presence of phage and plasmid carriage²⁷ or on β -lactamase production in the recipient.²⁸ In *S. epidermidis*, the presence of the chromosomal determinants for Cd and Hg resistance appears to play a role in determining recipient effectiveness for transduction of methicillin resistance.²³

The present study was undertaken to assess the incidence of resistance to metal ions in MRSA isolated at a London hospital between 1984 and 1986. The genetic location of the determinants was established by transduction and elimination of markers in selected isolates. The co-elimination of chromosomal markers with loss of methicillin resistance was also established.

Materials and methods

Bacterial strains, media and phages

Twenty-three isolates were consecutive but independent MRSA isolates from Guy's Hospital (GH isolates) obtained during 1984–1986; these included 10 isolates of the UK epidemic strain EMRSA-1. In outbreaks of infection only one isolate was included. The resistance phenotypes and phage types of 11 isolates studied for loss of methicillin resistance are shown in table I. Ten methicillin-sensitive *S. aureus* isolates obtained from Guy's patients in 1986 were also examined. Isolates provided by Dr R. R. Marples (Central Public Health Laboratory, Colindale Avenue, London) from various UK sources, included 10 EMRSA-1, nine other MRSA (OMRSA) and 10 methicillin-sensitive but antibiotic-resistant isolates (ORSA).¹⁹ Heterogeneous methicillin-resistant *S. aureus* strains CRF612 and 10442 were provided by Dr J. Richardson (CPHL). Recipients in transduction experiments were a rifampicin-resistant mutant of the restriction-deficient strain 80CR5,²⁹ a rifampicin-resistant mutant of strain SA113,³⁰ SA113R, provided by Dr J. Naidoo, or strain 8325–4 (RN 450), which is phage- and plasmid-free, provided by Dr R. Novick. The plasmid-free strain NCTC 8325 (8325N) was provided as a rifampicin-resistant mutant 8325NR by Dr J. Naidoo.

Bacteria were stored at -70°C in Nutrient Broth No. 2 (NB; Oxoid) containing glycerol 10% v/v. Cultures were maintained on Blood Agar (Tissue

Table I. Antimicrobial resistance and phage type of 11 consecutive MRSA isolates from Guy's Hospital

Isolate no.	Phage type (100RTD)	Resistance phenotype*
GH13†	85w	Mc Pc Pi Eb Gm Km Tm Tc Sm Em Tp Cd Hg
GH25†	85w	Mc Pc Gm Km Tm Tc Cm Sm Em Tp Cd Hg
GH28†	85w	Mc Pc Pi Eb Gm Km Tm Tc Cm Sm Em Tp Cd Hg
GH31†	85w	Mc Pc Pi Eb Gm Km Tm Tc Cm Sm Em Tp Cd Hg
GH19	NT or 29w/85	Mc Pc Pi Eb Gm Km Tm Tc Sm Nm Em Tp Cd Hg
GH30	NT or 29w/85	Mc Pc Pi Eb Gm Km Tm Tc Sm Nm Em Tp Cd Hg
GH27	85 (RTD)	Mc Pc Gm Km Tm Tc Sm Em Tp Cd Asa Asi Sb
GH32	NT	Mc Pc Gm Km Tm Tc Sm Em Fa Rf Cd Hg
GH23	52/52A/80/95/75/83A	Mc Pc Tc Sm Km Nm Em Fa Cd ^M
GH24	6/47/54/75/77/84/85/81 (RTD)	Mc Pc Gm Km Tm Tc Em Tp Rf Cd Asa Asi Sb
GH34	80 (RTD)	Mc Pc Gm Km Tm Tc ^L Cm Sm Nm Em Cd Hg Asa Asi Sb

^L, low level resistance (MIC 4 mg/L); ^M, moderate resistance (MIC 8–16 mg/L).

* See *Materials and methods*.

† EMRSA-1.

Culture Services) or Tryptic Soy Agar (TSA) prepared from Tryptic Soy Broth (TSB; Difco) solidified with Bacteriological Agar No. 1 (Oxoid) 1% w/v. Nutrient Agar (NA) was prepared from NB solidified with agar 1%.

Phages used for propagation on donor strains in transduction experiments were phage 80, phage 80 α (provided by Dr R. Novick), phage 83A (provided by Dr B. Berger-Bächi) and phage 24 obtained by UV induction of strain GH24. Phages were obtained by UV induction from strains GH19, GH30, GH32 and GH27 for testing as donors.

Susceptibility tests

Antibiotic disk sensitivity tests and tests for susceptibility to the NAB compounds Pi (provided by Rhone-Poulenc Ltd) and Eb (Sigma), were performed on Diagnostic Sensitivity Test Agar with lysed blood (Tissue Culture Services, Difco), with a semi-confluent inoculum prepared in phosphate-buffered saline. Antibiotic disks (Oxoid) were penicillin (Pc, 1.5 units) and (drug content in μ g), methicillin (Mc, 10), tetracycline (Tc, 10), chloramphenicol (Cm, 10), streptomycin (Sm, 10), neomycin (Nm, 10), kanamycin (Km, 5), gentamicin (Gm, 10), tobramycin (Tm, 10), erythromycin (Em, 5), clindamycin (Cl, 2), spectinomycin (Sp, 100), trimethoprim (Tp, 2.5), fusidic acid (Fa, 5 or 10), rifampicin (Rf, 2), vancomycin (Va, 30) and novobiocin (No, 5).

Disks containing Eb 50 μ g and Pi 50 μ g were tested by Stokes's method with *S. aureus* NCTC 6571 as the sensitive control. Sensitivity tests for metal ions were by Stokes's method on 0.3CY agar without added calcium³¹ employing strain 8325NR as the sensitive control because strain NCTC 6571 is resistant to Cd, Asa and Asi. Disks for metal-ion sensitivities were impregnated with 3CdSO₄ · 8H₂O (Cd) 20 μ g, phenyl mercuric nitrate (Hg) 0.13 μ g, inorganic mercury Hg(NO₃)₂ · H₂O 6.7 μ g or 10 μ g, Na₂ HAsO₄ · 7H₂O (Asa) 0.9 mg, NaAsO₂ (Asi) 0.33 mg, or KSbO C₄H₄O₆ (Sb) 12.5 μ g. Zinc (Zn) was tested as Zn (NO₃)₂ · 6H₂O.

For MIC determinations, 0.3CY agar without added calcium was used for arsenicals and Sb, NA for Cd, and peptone agar (Oxoid) for Hg. NA at pH 6.8 was used for the determination of the MICs of Zn.³² Doubling dilutions of the salts were used for MIC tests except with Zn, for which closely graded concentrations were used. In MIC tests, isolates were graded as resistant or sensitive respectively as follows: Cd $\geq$ 64 and $\leq$ 4.0 mg/L, inorganic Hg 1.25–5.0 and $\leq$ 0.63 mg/L, phenyl Hg 0.125–0.5 and $\leq$ 0.063 mg/L, Asa 2.0–4.0 and 0.25–1.0 g/L, Asi 0.25–0.5 and $\leq$ 0.06 g/L, Sb 0.2–0.4 and $\leq$ 0.1 g/L. Because Zn MIC values varied between experiments, susceptibility was expressed as a ratio⁹ of the MIC value for the test strain to that for the sensitive control strain 8325NR. Strains with moderate resistance to Cd (MIC 8–16 mg/L) were not reliably detected with disks containing 20,

10, 5 or 2.5 μ g of the metal salt. Methicillin MICs were determined on NA at an incubation temperature of 30°C, and at 30°C and 37°C to test for heterogeneity. Inocula were overnight TSB cultures diluted 1 in 100 in phosphate-buffered saline. End-points were read as an 100-fold or greater reduction in cfu at 24 or 48 h.

Loss of resistance

Sensitive variants of methicillin-resistant strains were isolated after growth in NB and storage at room temperature for 28 days or longer. Cultures diluted to give 100–200 colonies per plate were replicated on to NA and NA with methicillin 4 mg/L. Loss of Cd or Pi resistance was detected after growth for 24 h in NB at 43–44°C and replication on to agar containing Cd 5 or 10 mg/L or Pi 100 mg/L. Loss of β -lactamase production was detected³¹ by replication to starch 0.2% w/v agar, and the result was developed with a solution containing benzylpenicillin 600 mg in 20 ml of 0.1 M phosphate buffer, pH 6.0, with 64 mM iodine prepared in 120 mM potassium iodide.

Preparation of plasmid DNA

Plasmid DNA was isolated by a method based on that described by Stiffler *et al.*³³ (K.G.H. Dyke, personal communication). Strains were grown in CY broth³¹ to late logarithmic phase, harvested and washed with 10 mM cold EDTA, pH 7.0, treated with 2 volumes of ethanol:acetone (1:1) for 20 min at 4°C followed by addition of 4 volumes of 10 mM EDTA, pH 7.0, and centrifuged at 4°C. The deposit was dried in a dessicator and resuspended in 0.05 M EDTA with 0.1 M or 2.5 M NaCl, pH 7.0. Harvested cells were treated with lysostaphin (Sigma) 15 mg/L in 0.05 M EDTA, 0.1 M NaCl, pH 7.0, at 37°C until the start of lysis (c. 1 h). Lysis was completed at 4°C after the addition of 5 M NaCl (pH 7.0) 0.17 volumes, 0.5 M EDTA (pH 7.0) 0.09 volumes and SDS 2% in 0.7 M NaCl, 0.8 volumes. The cleared lysate (supernate) was obtained by centrifugation at 44 000 *g* at 4°C for 45 min. After treatment with TI RNAase 5–10 units/ml and pancreatic RNAase 50 mg/L, for 1 h at 37°C, followed by pronase 50 mg/L for 1 h at 37°C, DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of 96% ethanol at –20°C and stored at –70°C. The precipitate was deposited by centrifugation (16 000 *g*, 4°C, 20 min), dried in a desiccator, resuspended in 0.1 M NaCl, 0.05 M EDTA, pH 7.0, and stored at 4°C.

Transduction

Phages 83A, 80, 80 α and 24 were purified on the donor strain by single plaque purification and propagation by soft agar layer, or on the surface of lawn plates, by standard methods. Lysogenic donor strains insensitive to available phages were induced by exposure to UV light. Late logarithmic-phase cultures

Table II. Resistance to metal ions in methicillin-resistant and methicillin-sensitive *S. aureus*

Strain type	Number tested	Resistance phenotype of isolates					
		Cd ^M	Cd	CdHg	Cd, Asa, Asi, Sb	Cd, Hg, Asa, Asi, Sb	None
Methicillin-resistant							
EMRSA-1	20	0	1	19	0	0	0
OMRSA	22	6	2	10	1	3	0
Methicillin-sensitive	19	13*	4†	0	1	0	1

* One strain resistant to Asi, one to Asa and Asi, and one to Sb.

† Two strains with low level resistance to Asa.

Cd^M, moderate resistance to Cd.

(c. 3×10^8 cfu/ml) were washed in phosphate-buffered saline and exposed to a Hanovia monochromatic UV source for 15s at a distance of 23 cm. After the addition of 0.1 volume of $10 \times$ NB, irradiated cultures were grown with shaking for 2–3 h and left to lyse overnight. Phage lysates were harvested and filtered through a 0.45- μ m pore filter (Millipore). The propagating strain was tested for resistance. Transduction experiments based on methods previously described²³ used input ratios of 0.1–1.0. Selection for methicillin resistance was by plating on the surface of TSA plates containing methicillin 2, 3 or 4 mg/L. Recovery was unreliable in layer plates. Clavulanic acid (provided by Beecham Pharmaceuticals) was used at 1.5 mg/L in some experiments to inhibit background (recipient) growth. Plates were incubated at 30°C for 48 h. Transductants for Cd resistance were recovered on NA with CdSO₄. 8H₂O 4 or 10 mg/L and for tetracycline resistance on TSA containing tetracycline 20 mg/L. Recovery media contained 0.1 mM sodium citrate. Phage sterility was tested on selective media. In methicillin resistance transduction experiments, reconstruction experiments were included for recovery of the donor strain in the presence of the recipient under the same conditions as the transduction experiment.

Results

Resistance to metal ions

Results of tests for susceptibility to Cd, Hs, Asa, Asi and Sb in the methicillin-resistant and methicillin-sensitive isolates by disk and MIC methods are shown in table II. Strains resistant to phenyl Hg were also resistant to inorganic Hg. Zn sensitivity was tested by MIC. Amongst the MRSA strains, resistance to Cd and Hg was particularly characteristic of the EMRSA-1 isolates, which is consistent with the definition of this organism as a single strain. Moderate resistance to Cd (Cd^M; MIC 8–16 mg/L) occurred in six OMRSA strains and was common in the methicillin-sensitive isolates (13 out of 19). All of the Cd-resistant isolates, including those with moderate resistance, were 2–5-fold more resistant to Zn than the sensitive control strain 8325NR. Resistance to Asa, Asi and Sb in addition to Cd and Hg, was observed in only three MRSA isolates (table II). In disk tests for Asa

sensitivity, some strains, including EMRSA-1, showed a reduced zone size (> 10 mm reduction) compared with the control, which could not be confirmed by MIC tests with closely graded concentrations. These strains were grouped as Asa-sensitive.

Eleven consecutive, epidemiologically distinct isolates of MRSA (table I) were selected for further study. On the basis of phage type, seven of the isolates represented different strains. Four of the eleven isolates were EMRSA-1 (GH13, GH25, GH28 and GH31). Two other isolates (GH19 and GH30) were very similar in phage type, antimicrobial resistances and plasmid content (results not shown), and were considered to be one strain.

Resistance to methicillin

MIC values of methicillin for the 11 GH isolates ranged from 16 to 1024 mg/L at 30°C (table III). Single-colony cultures were used routinely for testing. Isolates with the highest level of resistance at 30°C were EMRSA-1. All isolates showed some degree of heterogeneity of resistance with MICs 2–8-fold lower at 30°C than at 37°C. On repeated testing, one EMRSA-1 isolate, GH25, underwent reversible changes in MIC and in the degree of heterogeneity expressed. On initial isolation, GH25 was homogeneous in resistance with similar MIC values at 30°C and 37°C of 512–1024 mg/L. When tested subsequently on several occasions, the initial homogeneity could not be confirmed but some heterogeneity of resistance was shown with MIC values of 512 mg/L at 30°C and 64 mg/L at 37°C. However, lower MICs

Table III. MICs of methicillin at 30°C and 37°C for 11 isolates of *S. aureus*

Number of isolates	MIC (mg/L) at	
	30°C	37°C
4*	512–1024	64–256
4	64–128	8–16
3	16–32	8–16
10442	128	16
CRF612	512–1024	16–32

* EMRSA-1.

of 32–64 mg/L at 37°C and 8–16 mg/L at 37°C were also obtained on some occasions. When retested, a minority of colonies (1 in 10 tested) from one of these cultures had reverted to the higher but heterogeneous level of resistance. Furthermore, one colony from the higher level, but heterogeneous, resistant strain showed more homogeneous resistance, similar to the original isolate with MICs of 1024 mg/L at 30°C and 512 mg/L at 37°C. These variants were unchanged in phage type. Passage on methicillin (4 mg/L) agar, which had been used for culture of MRSA in some cases, did not affect the levels of resistance or the degree of heterogeneity of these variants.

Efficiency of plating (EOP) on methicillin agar

Two strains, GH13 (EMRSA-1) and GH19, were responsible for outbreaks of infection. The degree of heterogeneity of methicillin resistance expressed was compared for these two strains by viable counts on methicillin agar at 30°C and 37°C (figure). The heterogeneous strains CRF 612 and 10442 were included for comparison. Strain GH13 was more resistant than strain GH19 at both 30°C and 37°C, as shown also in MIC tests. In addition, a higher proportion of cells of strain GH13 survived ($c. 10^{-3}$) at concentrations 2–4-fold above the MIC, compared with strain GH19 ($c. 10^{-5}$). The results for strain GH19 were very similar to those for strain 10442

(results not shown). Strain CRF 612 showed similar levels of survival to strain GH13 ($c. 10^{-3}$) but a greater degree of heterogeneity between the two temperatures than either strain GH13 or strain GH19. Based on the classification of Hartman and Tomasz³⁴ for the comparative survival of strains at 30°C and 37°C, but using 64 mg/L instead of 50 mg/L as the test concentration, strain GH13 expressed homogeneous resistance, strains GH19 and 10442 were heterogeneous and strain CRF 612 was temperature-sensitive heterogeneous in expression of resistance.

Loss and transduction of resistance to methicillin

Loss of resistance to methicillin occurred in 10 of the 11 MRSA isolates (all except strain GH30) in 0.2–5.0% of colonies screened after storage at room temperature. When tested for other changes in sensitivity (table IV) strains GH19, GH27, GH23, GH24 and GH34 remained unchanged, although some methicillin-sensitive variants of strain GH34 lost resistance to penicillin and metal ions which was associated with loss of a large plasmid (results not shown). However, EMRSA-1 isolates and strain GH32 consistently lost resistance to Cd and, in most cases, resistance to Hg with loss of methicillin resistance (table IV). Sensitive variants also showed increased sensitivity to Zn of 3–4-fold compared with the parent strain. Cd MIC values for the methicillin-sensitive

Figure. Efficiency of plating of methicillin-resistant strains GH13 and GH19 by viable counts on agar with increasing concentrations of methicillin after incubation for 48 h at 30°C or 37°C: strain GH13 at 30°C ○, and 37°C ●; strain GH19 at 30°C □, and 37°C ■; sensitive control CRF612 at 30°C △, and 37°C ▲.

Table IV. Co-elimination of markers with loss of methicillin or Cd resistance in MRSA

Isolate no.	Loss of resistance with loss of		Associated plasmid
	Mc	Cd	
GH13*	Cd Hg Tc	...	
GH25*	Cd Hg Tc	...	—
GH28*	Cd Hg Tc	...	—
GH31*	Cd Tc	...	—
GH19	0	Pc Pi Eb Cd Hg	+
GH30	NA (<0.04%)	Pc Pi Eb Cd Hg	+
GH27	0	Pc Cd Asa Asi Sb	+
GH32	Cd Tc or Cd Hg Tc	NA (<0.02%)	—
GH23	0	Pc Cd ^M	+
GH24	0	Pc Cd Asa Asi Sb	+
GH34	0	Pc Cd Hg Asa Asi Sb Sm Nm	+

* EMRSA-1 isolates.

... Not done.

NA, not applicable.

variants of EMRSA-1 isolates were generally lower (0.25–1.0 mg/L) than control strain 8325 (1–2 mg/L) (table V). Their increased susceptibility was also apparent in disk tests. MICs for the sensitive variants were 2–8 mg/L compared with 1–2 mg/L for the sensitive control strain 8325 NR. In most cases, MICs for the methicillin-sensitive variants were very similar when tested at 30°C or 37°C but strains GH23 and GH27 showed some degree of heterogeneity with 2–4-fold higher MIC values at 37°C.

It is likely that methicillin, Cd and Hg resistances were chromosomally encoded in EMRSA-1, because loss of these markers did not result in a change of plasmid profile (results not shown) and the loss of either of two plasmids carried by these strains—a large Pi plasmid (c. 30 kb) or a chloramphenicol-resistance plasmid (c. 4.3 kb)—did not affect the levels of resistance to the three markers. The EMRSA-1 isolates GH13 and GH31, and GH34, with their respective methicillin-sensitive variants were probed with a 2.6 kb *mec*-specific sequence derived from a 3.5 kb *mec*-associated region of DNA³⁵ following digestion of preparations of DNA with restriction enzyme *Bgl* II. Strain GH13 and its sensitive variant were also tested with a *mer-B* probe. This confirmed

Table V. MICs to tetracycline, Cd and Hg in methicillin-sensitive variant strains of EMRSA-1 and GH32

Strain	MIC (mg/L) of			
	Mc	Cd	Hg	Tc
Methicillin-resistant				
*EMRSA-1	512–1024	64	0.25	128
GH32	128	128	0.25	128
Methicillin-sensitive variants				
EMRSA-1	2–8	0.25–1.0	≤0.063 or 0.25	32
GH32	8	4–8	<0.03 or 0.125	32
Sensitive control	1–2	1.0	≤0.063	0.25

* Four isolates.

that DNA associated with the *mec* region had been deleted in the methicillin-sensitive variant strains (B. Berger-Bächi, personal communication).

Transduction of resistance to methicillin occurred with strain GH34 as donor and the non-lysogenic, chromosomal β -lactamase-producing strain 80CR5 as recipient. Transduction frequencies of (1.3×10^{-7}) – (6.5×10^{-8}) /pfu were obtained with UV-irradiated phage 80. MIC values for the methicillin transductants were substantially higher than for the donor strain with values of >1024 mg/L at 30°C and 1024 mg/L at 37°C for the transductants compared with 32–64 mg/L at 30°C and 8–16 mg/L at 37°C for the donor strain GH34. Passaging strain GH34 on methicillin (4 mg/L) agar, which was used for isolation and purification of transductants, did not affect the MIC. Methicillin-resistant transductants were not recovered with the remaining strains tested. Strains GH19, GH30, GH23, GH24 and GH27, when used as donors, failed to transduce methicillin resistance to strain 80CR5 at frequencies of $(<1.0 \times 10^{-9})$ – $(<5.0 \times 10^{-10})$ /pfu. Phage for transduction was 83A with strain GH23, 80 α with strain GH24 and was obtained by UV induction of the donor strain with strains GH19, GH30 and GH27. The effect on transduction of employing as recipient the lysogenic strain SA113, carrying the Cd-resistance, β -lactamase plasmid derived from the donor strains, was tested. No transductants were recovered at frequencies of $(<1.2 \times 10^{-9})$ – $(<4.2 \times 10^{-10})$ /pfu. Similarly with EMRSA-1 isolate GH13 as donor, no methicillin transductants were recovered with phage 24 and several different recipients. These included strain 80CR5 (transduction frequency $<2.3 \times 10^{-9}$ /pfu), a methicillin-sensitive variant of the lysogenic strain GH19 which had a β -lactamase, Hg Cd plasmid and a tetracycline plasmid ($<1.3 \times 10^{-9}$ /pfu) and a methicillin-sensitive variant of the EMRSA-1 strain GH31 which retained Hg resistance with loss of methicillin, Cd and high level tetracycline resistances ($<6.3 \times 10^{-10}$ /pfu). The latter recipient strain was used to test for the possibility that the Hg locus, which includes two IS-like sequences,³⁶ might contribute to the re-introduction of genes by homologous recombination.³⁷ Phage 24, used for transduction with EMRSA-1 as donor, had previously been shown to transduce the large Pi plasmid from strain GH13 at a low frequency of 1.0×10^{-9} /pfu and chromosomal erythromycin resistance at 2.1×10^{-5} /pfu.

Loss and transduction of resistance to Cd

In strains which lost methicillin-resistance independently of Cd resistance or other markers, the loss of Cd resistance was tested after growth at 43–44°C (table IV). Strain GH30, which retained methicillin resistance (2765 colonies tested), was also screened. With six isolates (GH19, GH30, GH23, GH24, GH27 and GH34), loss of Cd resistance was linked to loss of β -lactamase production and loss of a large plasmid

(c. 26–32.5 kb). In strain GH23, which had moderate resistance to cadmium, no other phenotypic change occurred. In the two similar isolates, GH19 and GH30, additional loss of resistance to Hg and to Pi and Eb occurred. In strains GH27 and GH24, resistance to Asa, Asi and Sb was co-eliminated and, in strain GH34, multiple resistance to metal ions, including Hg, and resistances to streptomycin and neomycin were lost with the large plasmid (table IV). Erythromycin resistance became inducible in this strain. In these six isolates, loss of the Cd resistance, β -lactamase-producing plasmid resulted in a reduced level of methicillin resistance of about 4-fold compared with the parent. In most cases, these variants still displayed heterogeneity when tested at 30°C and 37°C, suggesting that reduced methicillin resistance resulted from a plasmid-mediated character, which could be β -lactamase production. However, with loss from strains GH19 and GH30 of the plasmid which resulted in methicillin MIC values of 4–8 mg/L, heterogeneity was not clearly detected, suggesting that methicillin resistance may have been co-eliminated in these strains. Support for a plasmid location for Cd resistance was obtained with strains GH19, GH30, GH27, GH23, GH24 and GH34 by transduction and selection for Cd resistance (table VI). In each case, a large plasmid (c. 26–32.5 kb) appeared in the transductant. The markers co-transduced with strains GH19, GH30, GH23 and GH24 as donors and strain 8325-4 or strain SA113R as recipient were the same as those co-eliminated with Cd resistance, although some transductants with strain GH19 as donor lacked Pi and Eb resistance (table VI). When strain GH27 was employed as donor and strain 8325 as recipient, no transductants for Cd resistance were recovered ($< 5.0 \times 10^{-10}$ /pfu), but a low frequency of transduction was obtained (1.5×10^{-9} /pfu) when the recipient was a rifampicin resistant mutant of a Cd-sensitive variant of strain GH27 (GH2704). However, only about half of the transductants produced β -lactamase (table VI). When strain GH34 was the donor, transduction of Cd resistance confirmed the presence of neomycin and streptomycin resistance characters on the plasmid, but also showed co-transfer of kanamycin resistance

and of constitutive resistance to erythromycin and clindamycin. This strain (GH34), which phenotypically has constitutive resistance to erythromycin and clindamycin and resistance to spectinomycin, appears to have two loci for erythromycin resistance; this is being studied further.

Resistance to tetracycline and minocycline

Skinner *et al.*²⁵ described a 4-fold reduction in the level of tetracycline resistance in an epidemic MRSA strain (ANS46) from eastern Australia which had lost resistance to methicillin, Cd and Hg (ANS62); therefore, the MICs of tetracycline for methicillin-sensitive variants in this study were determined. The methicillin-sensitive variants of the four EMRSA-1 isolates, GH13, GH25, GH28 and GH31, and of strain GH32, which were the strains that showed co-elimination of Cd and usually Hg resistance with methicillin, showed a 4-fold reduction in resistance to tetracycline (MIC 32 mg/L) compared with the parent strains (128 mg/L) (table V). The low level of minocycline resistance (MIC 1–8 mg/L) which occurred in EMRSA-1 and strain GH32 was not altered in the methicillin-sensitive variants. A low level of resistance to minocycline also occurred in strains GH23, GH24 and GH27, but strains GH19 and GH30 were minocycline sensitive (MIC 0.25–0.5 mg/L). Four isolates GH19, GH30, GH24 and GH27, carried a tetracycline-resistance plasmid (c. 4.3 kb) as shown by loss and transduction of resistance (results not shown).

Discussion

The low incidence amongst strains in this study of multiple resistance to metal ions that included Cd, Hg and Asa, is consistent with a recent report of hospital isolates in the UK¹⁶ in which only methicillin-resistant strains were Hg resistant, and contrasts with earlier studies in which multiple metal-ion resistances, including Hg, were often coded on a β -lactamase plasmid.^{10,11} This trend is similar to that found in Danish hospitals where multiple resistance, including Hg, fell from 48% to 13% between 1967 and 1974 and was replaced by Cd- and Asa-resistant strains.¹⁷

The presence of chromosomal Cd and Hg resistance which is co-eliminated with methicillin resistance in the EMRSA-1 isolates from Guy's Hospital is further evidence for the similarity of this strain in the UK with the strain epidemic in eastern Australia. Like the Australian strain,²⁴ the methicillin-sensitive variants were slightly more resistant to methicillin (in this study 2–8 mg/L) than fully sensitive strains. The loss of Zn resistance with loss of high level Cd resistance suggests that *cadA*³⁸ is involved. Like the Australian strains isolated since 1970, the EMRSA-1 isolates in this study and strain GH32 were minocycline resistant (MIC 2–8 mg/L) and tetracycline resistant (MIC 32 mg/L) and had additional high level tetracycline

Table VI. Co-transduction of resistance markers with selection for Cd resistance in MRSA

Donor	Recipient	Transduction frequency (per pfu)	Phenotype of transductants
GH19	SA113R	1.7×10^{-7}	Pc Pi Eb Cd Hg or Pc Cd Hg
GH30	SA113R	8.1×10^{-7}	Pc Pi Eb Cd Hg
GH23	8325-4	4.4×10^{-7}	Pc Cd ^M
GH24	SA113R	5.1×10^{-7}	Pc Cd Asa Asi
GH27	GH2704	1.5×10^{-9}	Pc Cd Asa Asi or Cd Asa Asi
GH34	SA113R	c. 10^{-6}	Pc Km Nm Sm Em Cl Cd Hg Asa Asi

resistance (MIC 128 mg/L) which was lost with methicillin resistance. In the eastern Australian strains, this tetracycline-resistance determinant is equivalent to a complete insert of the tetracycline resistance plasmid pT181.^{25,26} Although there is no direct evidence that the same plasmid is present in the chromosome of the strains in this study, it has been found that MRSA from other sources in the UK have homology with pT181 (D. Dubin, personal communication). The residual tetracycline resistance and low level of minocycline resistance in EMRSA-1 and strain GH32, do not appear to conform to either the high level (*tmn*-3106)³⁹ or low level chromosomal resistances described by Asheshov⁴⁰ in *S. aureus*. Strain GH32, which is very similar to the EMRSA-1 isolates in the loss of chromosomal Cd, Hg and high level tetracycline resistance with methicillin, differs from these isolates in a number of other ways, including phage type and plasmid profile, in retaining a low level of Cd resistance (4 mg/L) in methicillin-sensitive variants, and in sensitivity to trimethoprim. It is also resistant to fusidic acid and to rifampicin, which did not occur in EMRSA-1 in this study and is not common in the UK.¹⁹ Strain GH32, which was isolated from a burns patient who had recently arrived from Greece, may have originated from outside the UK.

The clonal theory proposes that MRSA isolates are derived from a single strain on the basis of similarity in characteristics of early MRSA.^{41,42} These included stability and poor transferability of methicillin resistance and similarity in location of certain antibiotic resistances including plasmids for resistance to tetracycline and metal ions and for β -lactamase production. The recent epidemic strains in the UK^{5,19} and Australia differ from these earlier isolates in several respects. If they are derived from the earlier MRSA isolates, a considerable degree of change⁴ has occurred, which can be explained in part by the acquisition of antimicrobial resistance markers on the chromosome by transposition or insertion of these genes from other sources.¹⁵ The concentration of certain resistance characters in the region of methicillin resistance in MRSA, including resistance to Cd, Hg and tetracycline, suggests a role for this region in the build up of resistance on the chromosome. It has been suggested³⁷ that the insertion-sequence-like element (IS431) in the *mec* locus could provide a recombination site for resistances such as tetracycline and Hg which are also flanked by similar insertion sequences.²⁵ In further support of the clonal theory for the origin of methicillin-resistant strains, it has been suggested⁴³ that this

mec-associated DNA, which may provide integration sites, was acquired early in the evolution of the strain, and that modern MRSA isolates are derived from this organism.

The plasmid phenotypes of strains GH19 and GH30, for resistance to Pi, Eb, Cd and Hg and β -lactamase production has been described in several other countries, including epidemic strains in SE Asia⁶ and in Dublin hospitals.⁷ Strain GH19, which was also responsible for an outbreak of infection, has a tetracycline plasmid (c. 4.3 kb) and lacks chromosomal resistance to tetracycline and minocycline, making it more like the earlier classic isolates of MRSA.^{41,42}

The difficulty in transducing methicillin resistance (*mec*) recognised in other studies has been confirmed in this study. Some of the reasons for failure to transduce in this study could be in the use of phages which, although shown to transduce other markers, lack characteristics required for transduction of *mec*. Cohen and Sweeney⁴⁴ found that UV-induced lysogenic phage, which was used with four strains in this study, could not transduce methicillin if obtained directly from the donor strain (C5). Several reports have noted that phage 80, which was the phage involved in transduction of methicillin in this study, is more effective than transducing phages 53 and 29^{44,45} in transducing methicillin resistance. The enhanced effect of UV light on methicillin transduction suggests that homology is involved although *mec* does not have an allelic site in sensitive strains.^{35,46} If the large 30–40-kb *mec*-associated deletion found in an Australian epidemic strain^{24,25,36,47} has occurred in the MRSA in this study, regions of homology required for transduction may have been removed or the *mec*-containing fragment may be at the size limit for the transducing phage. In methicillin transduction with strain GH34 as donor, the similarity in phage type of the donor strain and strain PS80 from which the recipient strain 80CR5 is derived, may provide the homology necessary for transduction with this pair of strains. It was also noted that strain 80CR5 is non-lysogenic and plasmid free and has chromosomal β -lactamase, which are the conditions associated with transduction of methicillin-resistance by Stewart and Rosenblum,²⁸ also with phage 80.

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