

Characterisation and molecular cloning of the novel macrolide-streptogramin B resistance determinant from *Staphylococcus epidermidis*

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A total of 110 staphylococcal isolates from human skin were found to express a novel type of erythromycin resistance. The bacteria were resistant to 14-membered ring macrolides (MIC 32-128 mg/l) but were sensitive to 16-membered ring macrolides and lincosamides. Resistance to type B streptogramins was inducible by erythromycin. A similar phenotype, designated MS resistance, was previously described in clinical isolates of coagulase-negative staphylococci from the USA. In the UK, MS resistance is widely distributed in coagulase-negative staphylococci but was not detected in 100 erythromycin resistant clinical isolates of *Staphylococcus aureus*. Tests for susceptibility to a further 16 antibiotics failed to reveal any other selectable marker associated with the MS phenotype. Plasmid pattern analysis of 48 MS isolates showed considerable variability between strains and no common locus for the resistance determinant. In one strain of *S. epidermidis* co-resistance to tetracycline, penicillin and erythromycin (MS) was associated with a 31.5 kb plasmid, pUL5050 which replicated and expressed all three resistances when transformed into *S. aureus* RN4220. The MS resistance determinant was localised to a 1.9 kb fragment which was cloned on to the high-copy-number vector, pSK265. A constitutive mutant of *S. aureus* RN4220 containing the 1.9 kb fragment remained sensitive to clindamycin. This observation, together with the concentration-dependent induction (optimum 5 mg/l of erythromycin) of virginiamycin S resistance suggests that the MS phenotype is not due to altered expression of MLS resistance determinants (*erm* genes) but probably occurs *via* a different mechanism.

Introduction

Staphylococcal resistance to the macrolide antibiotic erythromycin usually involves co-resistance to both 14- and 16-membered ring macrolides and also to two other chemically distinct groups of antibiotics, the lincosamides and type B streptogramins (Weisblum, 1985). The three groups of antibiotics inhibit protein synthesis in prokaryotes by binding to the 50S ribosomal subunit. Resistance is conferred by N⁶ dimethylation of adenine residue 2058 of 23S rRNA (Lai & Weisblum, 1971; Skinner, Cunliffe & Schmidt, 1983). This causes reduced binding of all three classes of antibiotics giving rise to the co-resistant MLS_B phenotype. Three methylase genes have been identified in staphylococci. Inducible resistance is coded for by *erm* A (Murphy, 1985) and *erm* C (Horinouchi & Weisblum, 1982). Constitutive resistance is coded for by *erm* B (Novick & Murphy, 1985) and variants of *erm* C (Lampson & Parisi, 1986; Catchpole *et al.*,

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1988). A translational attenuation model has been proposed for the control of *erm* gene expression (Weisblum, 1985). In staphylococci low concentrations of erythromycin have been reported to be the most effective inducer of resistance (Weisblum & Demohn, 1969).

A novel phenotype which conferred inducible resistance to erythromycin and the streptogramin, pristinamycin I but not to lincomycin was reported several years ago in multiply-resistant strains of *Staphylococcus aureus* isolated in Hungary (Janosi & Ban, 1981). A similar phenotype (designated MS resistance) has been detected in the USA among clinical strains of coagulase-negative staphylococci (Jenssen *et al.*, 1987). The resistance appears to be distinct from MLS_B resistance and DNA from MS resistant isolates did not show homology to staphylococcal MLS_B determinants in dot blot analyses.

This paper describes the characterisation of 110 MS resistant isolates of coagulase-negative staphylococci isolated in the UK from normal human skin, and documents the cloning and expression of the MS resistance determinant from an *S. epidermidis* plasmid into *S. aureus* on the high-copy-number vector, pSK265.

Materials and methods

Bacterial strains and plasmids

Coagulase-negative staphylococci were isolated from the skin of acne patients attending the Leeds General Infirmary between January 1987 and July 1988. *S. aureus* RN4220, a restriction-minus derivative of the 8325-4 strain (Fairweather *et al.*, 1983) was used as the host in transformation and cloning experiments. All strains were grown at 37°C. Staphylococci were identified to species level by the method of Kloos and Schleifer (1975). The plasmids used are listed in Table I.

Antibiotic sensitivities and MICs

Strains demonstrating erythromycin resistance were screened for the MS phenotype by placing discs containing erythromycin, 5 µg, clindamycin, 2 µg and virginiamycin S or

Table I. Plasmids used in this study

Plasmid	Host	Description	Source or reference
pSK265	<i>S. aureus</i> RN4220	High copy number cloning vector, Cm ^R	Jones & Khan (1986)
pUL5050	<i>S. epidermidis</i> 968	MS ^R Tet ^R Pen ^R	This study
pUL5051	<i>S. aureus</i> RN4220	pSK265: 10.5 kb <i>Hind</i> III fragment of pUL5050, MS ^R Tet ^R Pen ^R Cm ^R	This study
pUL5054	<i>S. aureus</i> RN4220	pSK265: 1.9 kb <i>Sau</i> 3A fragment of pUL5050, MS ^R Tet ^R Pen ^R Cm ^R	This study

Cm, chloramphenicol; MS, 14-membered ring macrolides and type B streptogramins; Tet, tetracycline; Pen, penicillin; R, resistant; S, sensitive.

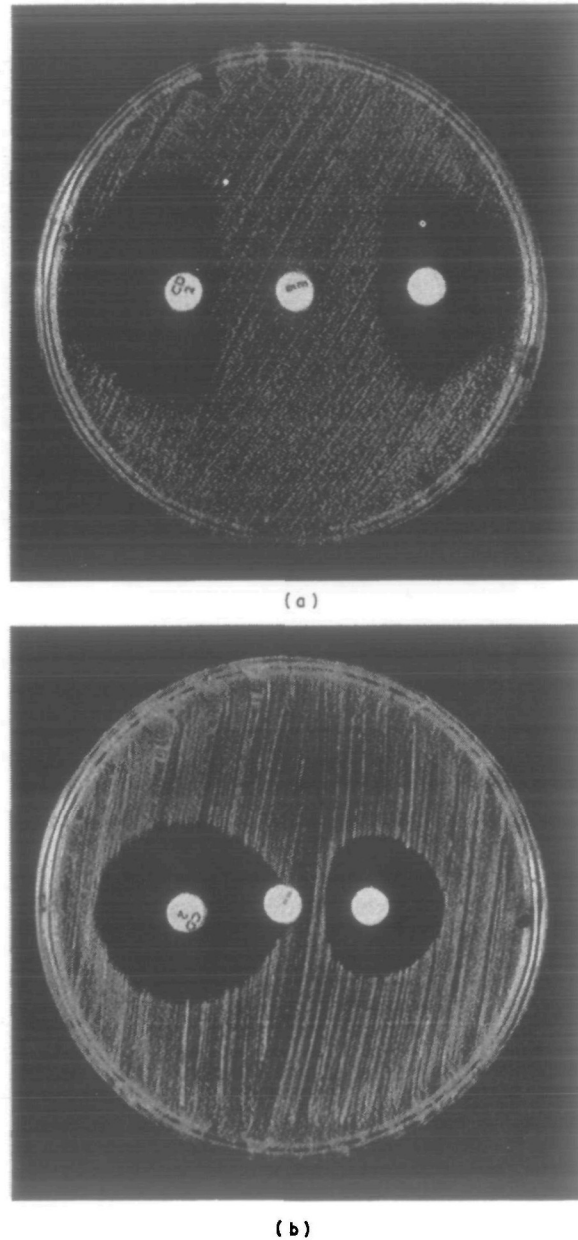


Figure 1. Erythromycin resistance phenotypes detected by disc diffusion. Isolate (a) shows classical MLS_B resistance as manifested by blunted zones adjacent to the erythromycin disc (E5, centre) for both clindamycin (CD2, left) and virginiamycin S (unmarked disc, right). In contrast, the MS resistant isolate (b) does not show blunting of the clindamycin zone next to the erythromycin disc.

osteogricin B, 50 µg on Iso-Sensitest agar (Oxoid) arranged as in Figure 1. Blunting only of the zone around the streptogramin B disc indicated an inducible MS phenotype while blunting around clindamycin and streptogramin B discs indicated an MLS_B phenotype. Strains exhibiting the MS phenotype were tested for sensitivity to a further

16 antibiotics using discs purchased from Diamed. MICs of eight MLS_B antibiotics for selected coagulase-negative strains and *S. aureus* transformed with plasmid pUL5050 and derived constructs were determined by agar dilution on Iso-Sensitest agar (ISA). An erythromycin-sensitive and an MLS resistant *S. epidermidis* were included as controls. Inocula were prepared by dilution of an overnight Iso-Sensitest broth culture to give a standard inoculum of 10⁴ cfu per spot delivered by a multi-point inoculator. The MIC of each antibiotic for each organism was recorded as the lowest concentration yielding no growth, or a barely visible haze as determined with the unaided eye. The MIC for induced cultures was found by adding 0.1 mg/l (or 5 mg/l, *vide infra*) erythromycin to overnight cultures and to MIC plates.

The antibiotics tested were purchased from Sigma Chemical Co., with the exceptions of clindamycin (a gift from Upjohn Ltd), josamycin (a gift from Dr P. M. Hawkey, this department) and the B type streptogramins (gifts from Glaxo Group Research). Clindamycin, lincomycin, spiramycin and oleandomycin were dissolved in distilled water. The remaining antibiotics were dissolved in ethanol and diluted in distilled water, with the exception of osteogricin B, which was dissolved in dimethyl sulphoxide. Streptogramin B discs were freshly prepared and stored at 4°C.

Induction of resistance in liquid culture

The growth of selected MS resistant organisms in the presence of MLS_B antibiotics was followed over a 24-h period. Overnight cultures of the test organisms were grown in the presence and absence of an inducing concentration of erythromycin (0.1 mg/l) in Tryptone Yeast Extract (TYE). A 0.5% inoculum was transferred to 30 ml of fresh TYE in a 250 ml conical flask with and without an inducing concentration of erythromycin. Cultures were either challenged with a higher concentration of a MLS_B antibiotic or left unchallenged. Challenge concentrations were 50 mg/l erythromycin, 20 mg/l clindamycin and 50 mg/l virginiamycin S for *S. epidermidis* or 25 mg/l for *S. aureus*. The optimum inducing concentration of erythromycin for virginiamycin S challenge was found to be 5 mg/l (see Figure 2). The flasks were incubated at 37°C on an orbital shaker and growth was assessed by measurement of optical density at 600 nm.

Small scale plasmid screening

Plasmid DNA was prepared from staphylococci by a modification of the method of Kieser (1984). Lysostaphin was substituted for lyszyme and used at a final concentration of 200 mg/l. Following isopropanol precipitation, samples were resuspended in 20 µl TE buffer (TrisHCl, 10 mM, pH 8; EDTA 1 mM, pH 8) and loaded on to agarose gels.

Large scale plasmid isolation

Staphylococcal plasmids were isolated by a modification of the cleared lysate/ caesium chloride method of Bibb, Freeman & Hopwood (1985), with substitution of lysostaphin for lysozyme at a final concentration of 100 mg/l. Following the clearing spin (20,000 g, 45 min) the supernatant was incubated at 37°C and treated successively with pancreatic ribonuclease, 100 mg/l (Sigma Chemical Co.) for 30 min and proteinase K, 50 mg/l (Boehringer-Mannheim) for 30 min, prior to polyethylene glycol precipitation.

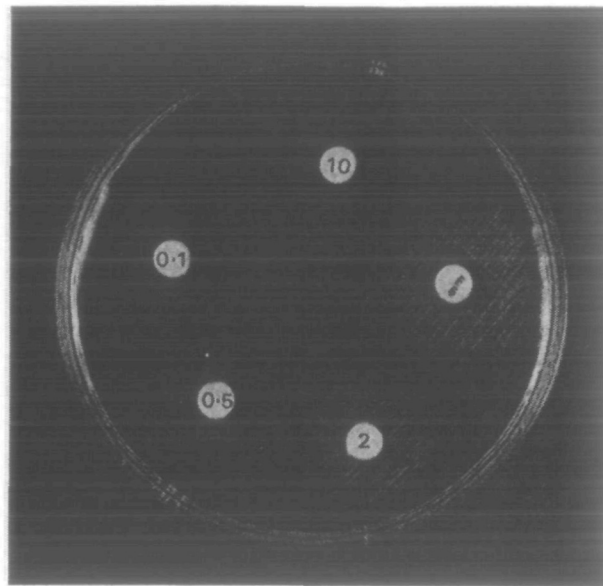


Figure 2. Optimization of the inducing concentration of erythromycin for virginiamycin S resistance. Plates containing 50 mg/l virginiamycin S were spread with a lawn of an MS resistant staphylococcus. Erythromycin-containing discs were placed on the surface. Concentrations were 10, 5, 2, 0.5, and 0.1 μg , as indicated on the discs. Visual assessment of the extent of growth revealed an optimum inducing concentration of 5 μg . This observation was confirmed in liquid culture.

Plasmid curing

Plasmid curing was carried out in broth culture at 42°C for 48 h. Cultures were plated on to non-selective media (ISA) and transferred to antibiotic-containing media with toothpicks. Sensitive isolates were screened for plasmid loss.

Transformation of S. aureus

S. aureus RN4220 was used as the host for transfer of plasmids by polyethylene glycol protoplast transformation (Gotz, Ahrne & Lindberg, 1981). Regeneration plates contained 5 mg/l erythromycin.

Restriction endonucleases and ligations

Restriction enzymes were purchased from Bethesda Research Laboratories. T4 ligase was obtained from Boehringer-Mannheim. Both were used according to manufacturers' instructions.

Results

Antibiotic susceptibility of MS resistant isolates

Staphylococcal isolates that demonstrated erythromycin resistance were tested for inducible resistance to lincosamides and streptogramin B antibiotics in disc tests (see Figure 1). Strains (110) that showed inducible resistance to type B streptogramins only

were classified as MS resistant and studied further. Erythromycin failed to induce resistance to the 16-membered ring macrolides, tylosin (5 µg), spiramycin (5 µg) and josamycin (2 µg) in disc tests on these organisms. Similarly the 16-membered ring macrolides did not induce resistance to clindamycin, virginiamycin S or osteogricin B. The 14-membered ring macrolide oleandomycin (10 µg) produced weak induction of streptogramin B resistance only.

The total of 110 MS resistant coagulase-negative staphylococcal isolates was found to comprise 56 *S. epidermidis*, 30 *S. hominis*, 10 *S. cohnii*, 5 *S. haemolyticus*, 1 *S. warneri*, 1 *S. capitis*, 1 *S. xylosum*, 2 *S. sciuri* and 4 untypable strains. The MS phenotype was not detected in any of 100 erythromycin-resistant *S. aureus* isolates. Screening with a panel of 16 antibiotics did not reveal a common co-resistance associated with the MS phenotype in the coagulase-negative staphylococci.

Determination of MICs for eight MLS antibiotics with and without induction confirmed the difference between MS and MLS resistance suggested by disc tests (Table II). Following induction, MS-resistant isolates demonstrated moderate resistance to the 14-membered ring macrolides, erythromycin (MIC 32–256 mg/l) and oleandomycin (MIC 16–128 mg/l) but remained sensitive to 16-membered ring macrolides and lincosamides. Resistance to the type B streptogramin, virginiamycin S showed a concentration-dependent induction which was optimal at 5 mg/l erythromycin. As expected, the MLS resistant control strain demonstrated high-level resistance (MIC ≥ 512 mg/l) to 14- and 16-membered ring macrolides, lincosamides and type B streptogramins, following induction with 0.1 mg/l erythromycin. The phenotype of MS resistant *S. cohnii* isolates differed slightly from that of the other coagulase-negative staphylococci. Erythromycin sensitive *S. cohnii* were resistant to 64 mg/l virginiamycin S and resistance apparently was not increased in MS-resistant strains. However, the induction of streptogramin B resistance could be demonstrated by the production of a blunted zone with 100 µg (as opposed to 50 µg) osteogricin B or virginiamycin S discs when placed adjacent to an erythromycin disc.

Genetics of MS resistance

Plasmid DNA was isolated from 48 strains of MS-resistant staphylococci by the small scale plasmid extraction procedure. DNA was analysed on agarose gels with uncut plasmid size markers from *Escherichia coli* V517 (Macrina *et al.*, 1978). Analysis of these preparations failed to identify a common plasmid present in all isolates. However, the majority of isolates (79%) possessed at least one large plasmid of 30–32 kb. In addition, 50% of isolates carried a small plasmid of 4–4.5 kb, which was larger than pE194, the well characterised 3.7 kb *S. aureus* MLS resistance plasmid. An even smaller plasmid of approximately 2 kb was present in 42% of isolates. All but five isolates possessed at least one of these three plasmids, which represent possible loci for the MS resistance determinant. One strain of *S. hominis* was found to be plasmid-free even upon repeated testing.

Three MS-resistant strains were subjected to plasmid curing experiments. The loss of the MS phenotype and co-resistance to penicillin was associated with the loss of a 30–32 kb plasmid in all three strains. *S. epidermidis* 968 contained only a single large plasmid (pUL5050) and demonstrated resistance to erythromycin, tetracycline and penicillin, all of which were lost during curing. In order to confirm the association between the plasmid and MS-resistance, better define the phenotype, and clone the resistance gene, plasmid DNA was isolated from *S. epidermidis* 968 by caesium chloride

Table II. MICs of MLS_B antibiotics for *MS* resistant staphylococci

Inducer ^a :	ERY		OLE		TYL		MIC (mg/l) SPI		JOS		CLD		LIN		VIRG _S	
	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
<i>S. epidermidis</i> 968	64	256	64	64	0.5	1.0	2	4	0.25	1.00	0.06	0.06	0.25	0.25	16	> 256 ^b
<i>S. epidermidis</i> 1187	64	128	64	128	0.5	0.5	1	2	0.25	0.25	0.06	0.06	0.25	0.25	4	> 256 ^b
<i>S. epidermidis</i> 1214	128	128	128	128	0.5	1.0	2	4	0.25	1.00	0.06	0.06	0.50	0.50	8	> 256 ^b
<i>S. epidermidis</i> 1481	128	256	64	128	1.0	1.0	2	4	0.25	0.50	0.06	0.06	0.50	0.50	8	> 256 ^b
<i>S. hominis</i> 3376	32	64	64	64	1.0	1.0	2	2	0.50	1.00	0.06	0.06	0.50	0.50	16	> 256 ^b
<i>S. cohnii</i> 1434	32	32	16	16	1.0	2.0	4	4	1.00	1.00	0.25	0.06	4.00	1.00	64	64 ^b
Sensitive																
<i>S. epidermidis</i> NCTC 11047	0.25	0.125	1	0.5	0.5	0.5	2	1	0.50	0.25	0.06	0.06	0.5	0.06	8	NA
MLS_B resistant																
<i>S. epidermidis</i> 1231	≥ 512	≥ 512	≥ 512	≥ 512	1.0	64.0	32	≥ 512	1.00	≥ 512	0.25	≥ 512	1.0	≥ 512	8	≥ 512

ERY, erythromycin; OLE, oleandomycin; TYL, tylosin; SPI, spiramycin; JOS, josamycin; CLD, clindamycin; LIN, lincomycin; VIRG_S, virginiamycin S; NA, not applicable.

^aInducing concentration of erythromycin was 0.1 mg/l.

^bVIRG_S resistance was induced with 5 mg/l erythromycin.

density gradient centrifugation. This was then transformed into a sensitive plasmid free recipient *S. aureus* RN4220. Agarose gel electrophoresis of erythromycin resistant transformants confirmed the presence of pUL5050. The phenotype conferred by pUL5050 in *S. aureus* was identical in disc tests to the phenotype of *S. epidermidis* 968 and MIC values (see Table III) were very similar.

Analysis of restriction fragments of pUL5050 gave a size of approximately 31.5 kb. In order to study the resistance gene further it was cloned from pUL5050 on a 10.5 kb *Hind*III fragment on to the high-copy-number *S. aureus* vector, pSK265. Penicillin resistance was also expressed by the fragment. The size of the insert was subsequently reduced by partial digestion of the 10.5 kb fragment with the restriction enzyme *Sau*3A. Suitably sized bands (1–4 kb) were excised from Bio-Rad ultra-pure DNA grade agarose and ligated to pSK265 cut with *Bam*HI. Transformation of *S. aureus* RN4220 and screening of erythromycin-resistant transformants revealed a 1.9 kb insert in pSK265 (pUL5054) which conferred the MS phenotype. MIC determinations (see Table III) indicated that possession of the gene on a high-copy-number plasmid did not alter the MIC of erythromycin or virginiamycin S. A mutant of *S. aureus* RN4220 containing pUL5054 was isolated from around a virginiamycin S disc and was shown to demonstrate constitutive resistance to virginiamycin S in disc tests and MIC determinations (see Table III).

To study the inducible nature of the MS resistance determinant, growth over a 24-hour period was monitored with and without induction (Figure 3). It was determined during preliminary experiments (see Figure 2) that induction of virginiamycin S resistance occurred at an optimum erythromycin concentration of 5 mg/l. Therefore, this concentration was used to induce resistance to virginiamycin S. In liquid culture, *S. epidermidis* 968 demonstrated inducible resistance to erythromycin and virginiamycin S but not clindamycin (Figure 3). However, after an extended lag period the isolate grew in 50 mg/l erythromycin without prior induction. A similar pattern of growth was produced by *S. aureus* RN4220 containing the wild type plasmid pUL5050. In contrast, *S. aureus* containing the multicopy plasmid pUL5054 (containing the 1.9 kb fragment of pUL5050) demonstrated constitutive resistance to erythromycin but inducible resistance to virginiamycin S. The mutant obtained from *S. aureus* RN4220 containing pUL5054 demonstrated constitutive resistance to both erythromycin and virginiamycin S. No growth of the mutant was observed in media containing clindamycin with or without erythromycin induction.

Thirty-five MS resistant coagulase-negative staphylococcal isolates and *S. aureus* transformed with pUL5054 failed to show any inactivating enzyme activity in a

Table III. MICs of transformed *S. aureus* and derivatives

Plasmid	Inducer:	MIC (mg/l)			
		ERY		VIRG _s	
		–	+	–	+
None		0.5	0.25	8	4
pUL5050		128	128	8	> 256
pUL5054		128	128	8	> 256
pUL5054-constitutive mutant		128	128	128	> 256

Abbreviations, see Table II. For concentrations of inducer, see Table II.

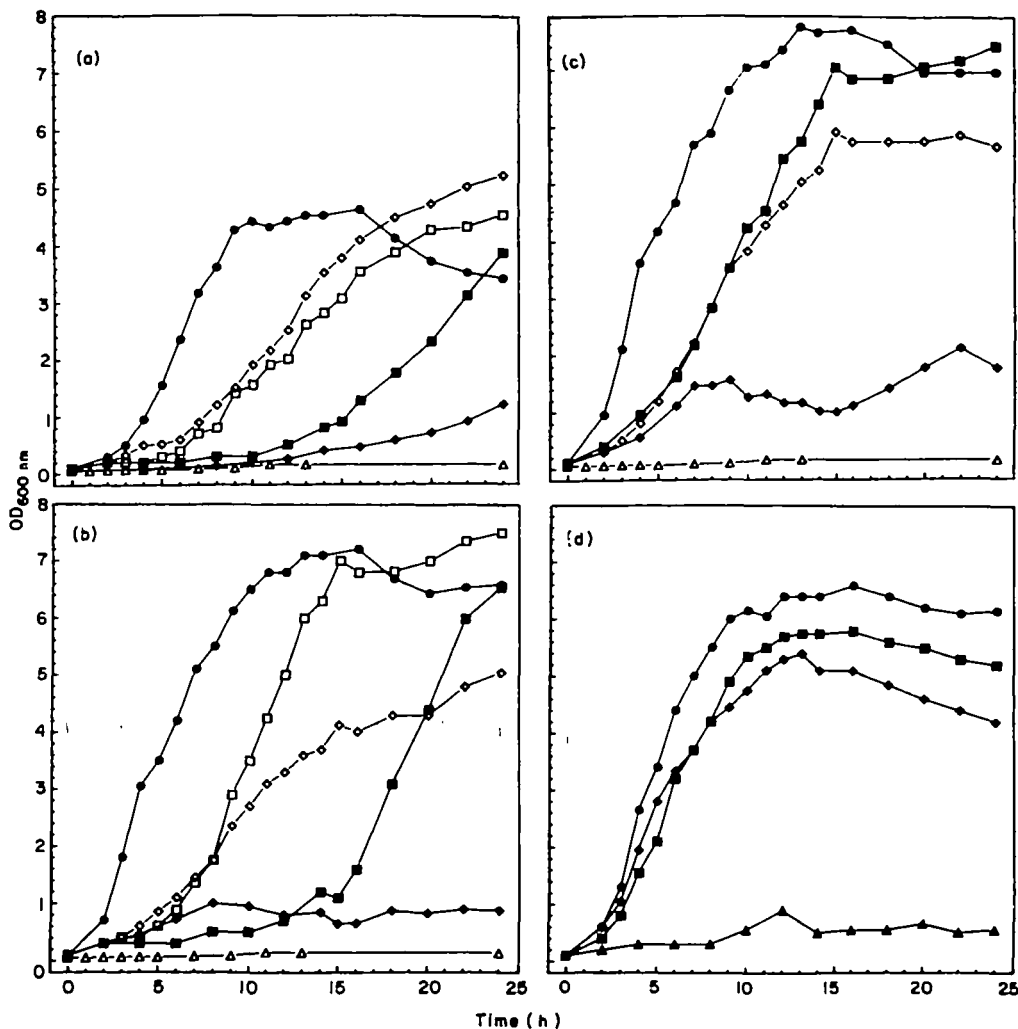


Figure 3. Growth in liquid culture of MS-resistant staphylococci. (a) *S. epidermidis* 968; (b) *S. aureus* RN4220 containing pUL5050; (c) *S. aureus* RN4220 containing pUL5054; (d) constitutive mutant of *S. aureus* RN4220; (e) cultures were challenged with MLS₂ antibiotics with or without prior induction. Closed symbols refer to uninduced cultures, open symbols refer to erythromycin-induced cultures. No challenge (●); erythromycin (■); clindamycin (▲); virginiamycin S (◆).

modified Gots' test (Haight & Finland, 1952) in which 0.1 mg/l erythromycin was substituted for penicillin, even after prolonged incubation (four days at 37°C).

Discussion

A novel phenotype of erythromycin resistance was originally reported in multiply resistant isolates of *S. aureus* from Hungary (Janosi & Ban, 1981). A similar phenotype, designated MS resistance, was reported six years later in clinical isolates of coagulase-staphylococci in the USA (Jenssen *et al.*, 1987). In the UK we have isolated MS resistant isolates of coagulase-negative staphylococci from the surface of human skin

where they are present in the majority of subjects as part of the normal commensal flora. The strains were not associated with infection. In accordance with the results of Jenssen *et al.* (1987), MS resistance was found to be widespread in isolates of all the common species of coagulase-negative staphylococci. The MS phenotype differs from classical MLS resistance by not demonstrating cross-resistance to the lincosamides and 16-membered ring macrolides even after induction. MS resistance was found to be expressed in a slightly different form in *S. cohnii* to the other species. Sensitive isolates of this species possess a natural resistance to type B streptogramins (MIC 64 mg/l). The inducible nature of the resistance could be demonstrated by increasing the concentration of virginiamycin S or osteogricin B from 50 to 100 µg per disc. It is probable that the MS phenotype is superimposed on the natural resistance in this species. The MS phenotype has so far not been detected in *S. aureus* in Leeds, although we have shown that the *S. epidermidis* determinant is expressed in an *S. aureus* background.

Plasmid screening did not reveal a common locus for the MS resistance determinant. MLS genes are usually located on small plasmids (< 4 kb) such as pE194 (Horinouchi & Weisblum, 1982) and pNE131 (Parisi *et al.*, 1981). However, MS resistance genes may frequently be located on large (30–32 kb) plasmids, such as pUL5050, which were present in 79% of strains examined and, in the three strains tested, were shown to be co-cured with MS and penicillin resistance. The MS resistant determinant in *S. aureus* was shown by Janosi & Ban (1981) to be located on a 23.5 kb plasmid in four out of five strains examined. The plasmids additionally coded for resistance to penicillin and the heavy metals, cadmium and mercury. Although there seems to be a strong association with penicillin resistance, this is not always the case. Fifteen percent (16 out of 110) of the Leeds isolates did not demonstrate penicillin resistance. Lampson, von David & Parisi (1986) found a large 26.5 kb plasmid, pNE24 associated with resistance to 14-membered ring macrolides in *S. epidermidis*. In contrast to pUL5050, which encodes resistance to penicillin and tetracycline in addition to erythromycin, no other antibiotic resistance markers were detected on this plasmid. Erythromycin resistance encoded by pNE24 was reported to be non-inducible and the plasmid apparently conferred no resistance to type B streptogramins. However, in view of the finding that virginiamycin S resistance due to pUL5050 required induction with 5 mg/l of erythromycin, it is possible that Lampson *et al.* (1986) did not identify optimal inducing conditions for pNE24. The relationship between the resistances conferred by these two plasmids, is, therefore, unclear. The optimum inducing concentration of erythromycin for the expression of streptogramin B resistance conferred by pUL5050 is 50–100 times higher than the optimum for induction of MLS resistance (Weisblum, 1985). Following induction, MLS genes confer resistance to > 512 mg/l of 14- and 16-membered ring macrolides, lincosamides and B type streptogramins. By comparison, fully induced MS resistance genes confer only a moderate level of resistance to 14-membered ring macrolides (≥ 128 mg/l) and B type streptogramins (≥ 256 mg/l). A number of MLS antibiotics other than erythromycin were tested as inducers but none was found to turn on the expression of resistance to 16-membered ring macrolides or lincosamides.

The growth kinetics of MS resistant staphylococci in liquid culture confirmed the inducible nature of the resistance encoded by pUL5050. Transfer of the determinant to a high-copy-number vector to produce pUL5054 led to apparent constitutive expression of erythromycin resistance but virginiamycin S resistance remained inducible.

Mutants spontaneously arising in *S. aureus* containing pUL5054 expressed both

erythromycin and virginiamycin S resistance constitutively but remained sensitive to clindamycin. Therefore, although there are certain similarities between MS and MLS resistant staphylococci there are insufficient grounds for believing that the former is due to altered expression of MLS resistance determinants. It is difficult to understand how methylation of 23S ribosomal RNA at position 2058 could fail to protect against clindamycin challenge whatever alteration may have occurred in the attenuator region of the *erm* gene. The relationship between MS and MLS resistant staphylococci at the phenotype level is thus unlikely to exist at the genotype level. The dose-dependent induction of streptogramin S resistance in MS resistant strains may in part be explained by the greater affinity of erythromycin for the known overlapping binding site in 50S ribosomal subunits (Moazed & Noller, 1987). Although distinct from MLS resistance, it is likely that MS resistance is due to a ribosomal modification of some kind, rather than mechanisms such as efflux, inactivation *etc.*, which are inconsistent with the observed characteristics of MS resistant strains. Such mechanisms fail to explain the prolonged lag phase or inducible resistance to the chemically-unrelated streptogramin B antibiotics. A single enzyme which could inactivate such structurally diverse antibiotics is also unlikely and no inactivation of erythromycin was detected by over 40 isolates examined. Work is currently in progress to sequence the 1.9 kb insert of pUL5054 in order to identify the MS resistance gene product and the means by which it operates.

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