Journal of Medical Microbiology (2008), **57**, 364–372 DOI 10.1099/jmm.0.47621-0

Analysis of meticillin-susceptible and meticillin-resistant biofilm-forming *Staphylococcus aureus* from catheter infections isolated in a large Italian hospital

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Several characteristics were analysed in 37 *Staphylococcus aureus* isolates from nosocomial catheter infections: the PFGE profile after *Sma*I digestion of chromosomal DNA, the ability to form a biofilm on a polystyrene surface, antibiotic susceptibility patterns (penicillin, oxacillin, erythromycin, tetracycline, clindamycin, telithromycin, gentamicin, ciprofloxacin, quinupristin/dalfopristin, rifampicin, vancomycin and linezolid), and the presence of genetic determinants of antibiotic resistance and biofilm formation. All strains but three (92 %) were able to grow on a plastic surface as a biofilm. An almost complete association was found between phenotypes and genotypic traits of antibiotic resistance, whilst PFGE profiling showed the highly polyclonal composition of the set of strains under study. Sixteen isolates (43 %) were meticillin-resistant and were subjected to staphylococcal cassette chromosome *mec* (SCC*mec*) and cassette chromosome recombinase (*ccr*) complex type determination by multiplex PCR. Only a subgroup of six strains belonged to the archaic clone PFGE type and bore the SCC*mec*/*ccrAB* type I structure. Among the remaining strains some presented small rearrangements of the SCC*mec*/*ccrAB* genetic locus, whilst others could barely be traced back to a known structural type. These observations suggest that, at the local level and at a particular site of infection, *S. aureus* may show great genetic variability and escape the general rule of expansion of the *S. aureus* pandemic clones.

Received 10 September 2007
Accepted 26 November 2007

INTRODUCTION

The coagulase-positive species *Staphylococcus aureus* is well documented as an important nosocomial pathogen that causes various skin infections, bacteraemia, pneumonia, osteomyelitis, endocarditis, myocarditis, meningitis and abscesses at different sites. Since the 1980s, meticillin-resistant *S. aureus* (MRSA) has emerged as a major clinical and epidemiological problem in hospitals (Gould, 2005). A distinctive feature of MRSA strains is their resistance not only to all β-lactam antibiotics, but also to a wide range of other antimicrobials (Rice, 2006), which makes MRSA infections difficult to manage and costly to treat (Gould, 2006). Moreover, *S. aureus* and its relative *Staphylococcus epidermidis* are frequently the cause of bacteraemia related to foreign bodies and indwelling medical devices (Donlan & Costerton, 2002). On these inert surfaces the bacteria are able to grow as biofilms, which are refractory to antimicrobial agents (Donlan & Costerton, 2002). Removal of the infected device is often the only possible clinical solution, thus increasing the trauma to the patient and the cost of treatment. Various genes have been implicated in the onset and maintenance of biofilms by staphylococci. Among these, the most extensively studied are *icaA* and *icaD*. Their products are responsible for the synthesis of polysaccharide intercellular adhesin, a major component of the exopolysaccharide matrix that embeds bacterial cells in the biofilm (Rohde *et al.*, 2001). Also, the products of *pls* (Savolainen *et al.*, 2001), which encodes a surface protein, and *atl* (Biswas *et al.*, 2006), which encodes an autolysin, have been implicated, to various extents, in the formation and structuring of biofilms.

Among intravascular devices, the use of central venous catheters (CVCs) is frequently followed by both local and systemic complications, including septic thrombophlebitis, endocarditis, metastatic infections and bacteraemias (Maki & Mermel, 1998). In particular, it is estimated that over

Abbreviations: CIP, ciprofloxacin; CLI, clindamycin; CVC, central venous catheter; ERY, erythromycin; GEN, gentamicin; MRSA, meticillin-resistant *Staphylococcus aureus*; MSSA, meticillin-susceptible *Staphylococcus aureus*; OXA, oxacillin; PEN, penicillin; QD, quinupristin/dalfopristin; RIF, rifampicin; TEL, telithromycin; TET, tetracycline.

80 % of all catheter-related bloodstream infections are associated with CVCs (van Belkum, 2000).

MRSA strains can be investigated by various typing schemes. They appear to be closely related genetically (Enright *et al.*, 2000). Many molecular epidemiology studies clearly indicate that the massive geographical spread of MRSA results from the dissemination of relatively few highly epidemic clones (Oliveira *et al.*, 2001).

The first stage in the emergence of MRSA is acquisition of the *mecA* gene and associated *mec* DNA by meticillin-susceptible *S. aureus* (MSSA), and their integration into its chromosome. The *mecA* gene encodes an extra penicillin-binding protein PBP 2a or PBP 2′ that allows cell wall synthesis to continue despite inactivation of native penicillin-binding proteins. There are five genetic classes of the *mec* gene complex, each consisting of an intact copy of *mecA*, a copy of IS*431mec* and, when present, complete or truncated *mec* regulatory genes *mecI* and *mecR1* (Hiramatsu *et al.*, 2001). The *mec* gene complex is carried on a mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*). This locus also contains a cassette chromosome recombinase (*ccr*) complex, consisting of the *ccr* genes *ccrA* and *ccrB* in combination (*ccrAB*) or the *ccr*C gene alone, in addition to the adjacent ORFs (Ito *et al.*, 2004). The rest of the SCC*mec* element, which lies outside the *ccr* and *mec* complex, is known as the junkyard or J region. It may contain various antibiotic resistance genes. To date, five types of SCC*mec* element (I–V) and a number of subvariants have been characterized (Grundmann *et al.*, 2006).

The origin of these elements, as well as the number of times that these foreign pieces of DNA have entered the *S. aureus* species, and the mechanisms of their acquisition remain substantially unknown and are the subject of lively scientific debate (Deurenberg *et al.*, 2007). Recent studies of the evolutionary history of MRSA suggest multiple introductions of the five SCC*mec* elements into MSSA strains with the same sequence type, indicating that horizontal transfer of *mec* genes is relatively frequent within *S. aureus* (Lim *et al.*, 2003; Oliveira *et al.*, 2002; Robinson & Enright, 2003).

In this work, we analysed a group of *S. aureus* strains isolated from central intravenous catheters in patients presenting clinical signs of bacteraemia and positive blood cultures. The antibiotic resistance profile was determined, along with the ability to form a biofilm on inert surfaces, followed by PCR identification of the genetic determinants of resistance/biofilm production and *Sma*I macrorestriction followed by PFGE. The SCC*mec* elements of the identified MSRA strains were subsequently defined and related to the other investigated characteristics.

METHODS

Bacterial strains. In the General Hospital of Perugia (Perugia, Italy), between September 2003 and May 2004, patients with removable CVCs who showed clinical manifestations of bloodstream infection were considered. Isolates were taken from long-dwelling catheters with a duration of placement of >1 week that yielded positive to semi-quantitative cultures (⩾15 c.f.u.) (Mermel *et al.*, 2001). Two independent isolates from different samples taken from the same patient were obtained and identified using a Vitek 2 system instrument. Thirty-seven single *S. aureus* isolates were collected. *S. epidermidis* ATCC 35984 (complete genome sequence: GenBank accession no. NC_002976) was used as a biofilm-positive reference strain together with *S. epidermidis* 1457 (Mack *et al.*, 1994). The *S. epidermidis* transposon mutant 1457-M11 was used as a biofilm-negative strain (Mack *et al.*, 1994). Staphylococcal pandemic reference strains used in *Sma*I macrorestriction profiling by PFGE and in the determination of the SCC*mec* type were (each strain is reported as 'name of strain-SCC*mec* type-multilocus sequence typing sequence type'): COL-I-st250, HPV107-IA-st247, BK2464-II-st5, HUSA304-III-st239, HSJ216-IIIA-st239 (Oliveira *et al.*, 2002), PER34-IA-st250 (Dominguez *et al.*, 1994), JP1-II-st5 (Aires de Sousa *et al.*, 2000), HU25-IIIA-st239 (Teixeira *et al.*, 1995), PL72-IV-st5 (Leski *et al.*, 1998), BM18-IV-st5 (Roberts *et al.*, 1998), BAR2529-V-st8 (Kreiswirth *et al.*, 1993) and UK13136-I-250 (Crisostomo *et al.*, 2001). Isolated bacteria and reference strains were stored at −80 °C.

Antibiotic susceptibility. The determination of MICs was performed in accordance with the Clinical Laboratory Standards Institute guidelines (CLSI, 2005). Penicillin (PEN), oxacillin (OXA), erythromycin (ERY), tetracycline (TET), clindamycin (CLI), telithromycin (TEL), gentamicin (GEN), ciprofloxacin (CIP), quinupristin/dalfopristin (QD) and rifampicin (RIF) (Oxoid) were tested by the disc diffusion method. The Etest and microdilution methods were used for linezolid (AB Biodisk) and vancomycin (Sigma), respectively.

Biofilm formation. The ability to form a biofilm was evaluated using the crystal violet staining test according to a previously described protocol (Petrelli *et al.*, 2006). This method measures the crystal violet stain retained by cultured bacterial cells adhering to the bottom of microtitre plate wells after repetitive cycles of washing. All strains that showed a low biofilm formation capacity with the above described crystal violet method were retested following the protocol described by Christensen *et al.* (1985).

PCR screening of the genetic determinants of antibiotic resistance, adhesion and biofilm formation. All amplification reactions were prepared in a 25 μl volume containing: 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.25 mM $MgCl_2$, 100 μM each dATP, dCTP, dGTP and dTTP, 1 μM each oligonucleotide primer, 1 U *Taq* polymerase and 200 ng template DNA. All strains were investigated to detect the presence of genes associated with the screened antibiotic resistances, namely: *blaZ* (PEN resistance); *mecA* (OXA resistance); *tetK*, *tetM*, *tetL* and *tetO* (TET resistance); *ermA*, *ermB*, *ermC*, *msrA*, *vatA*, *vatB*, *vatC*, *vgaA* and *vgaB* (ERY, CLI, TEL and QD resistance); and *aac(6′)–aph(2″)* (GEN resistance).

Oligonucleotide primer sequences for the detection of antibiotic resistance-associated and biofilm-associated genes are reported in Table 1.

PCR typing of the SCC*mec* element. Characterization of structural variations in the SCC*mec* element was achieved by means of a multiplex PCR protocol following published procedures (Oliveira & Lencastre, 2002). As additional confirmation, or when at least one of the structural features typical of a particular SCC*mec* type was not identified by this methodology, PCR amplification of the *ccr* complex was performed (Zhang *et al.*, 2005).

Macrorestriction and PFGE. The general conditions for macrorestriction with *Sma*I and PFGE have been described by Chung *et al.*

Table 1. PCR target genes and primers used in this work

Gene	Primer		Reference
	No.	Sequence (5′→3′)	
*ica*A	1	ACACTTGCTGGCGCAGTCAA	Rohde *et al.* (2001)
	2	TGTTGGATGTTGGTTCCAGA	
*ica*D	3	ATGGTCAAGCCCAGACAGAG	Rohde *et al.* (2001)
	4	TTGCTTTAAACATTGAAAATACT	
atl	5	CTTCAGCACAACCAAGATC	This work*
	6	GGTTACCGACTGCACCGTCAC	
pls	7	GTAATACAACAGGAGCAGATGG	This work*
	8	GTAGCTTTCCATGTTTTTCCTG	
*mec*A	9	AACAGGTGAATTATTAGCACTTGTAAG	Martineau *et al.* (2000)
	10	ATTGCTGTTAATATTTTTTGAGTTGAA	
blaZ	11	ACTTCAACACCTGCTGCTTTC	Martineau *et al.* (2000)
	12	TGACCACTTTTATCAGCAACC	
ermA	13	TATCTTATCGTTGAGAAGGGATT	Martineau *et al.* (2000)
	14	CTACACTTGGCTTAGGATGAAA	
ermB	15	CTATCTGATTGTTGAAGAAGGATT	Martineau *et al.* (2000)
	16	GTTTACTCTTGGTTTAGGATGAAA	
ermC	17	CTTGTTGATCACGATAATTTCC	Martineau *et al.* (2000)
	18	ATCTTTTAGCAAACCCGTATTC	
msrA	19	TCCAATCATTGCACAAAATC	Martineau *et al.* (2000)
	20	AATTCCCTCTATTTGGTGGT	
tetK	21	TCGATAGGAACAGCAGTA	Ng *et al.* (2001)
	22	CAGCAGATCCTACTCCTT	
tetL	23	TCGTTAGCGTGCTGTCATTC	Ng *et al.* (2001)
	24	GTATCCCACCAATGTAGCCG	
tetM	25	GTGGACAAAGGTACAACGAG	Ng *et al.* (2001)
	26	CGGTAAAGTTCGTCACACAC	
tetO	27	AACTTAGGCATTCTGGCTCAC	Ng *et al.* (2001)
	28	TCCCACTGTTCCATATCGTCA	
vatA	29	CAATGACCATGGACCTGATC	Werner *et al.* (2001)
	30	AGCATTTCGATATCTCC	
vatB	31	CCTGATCCAAATAGCATATATCC	Werner *et al.* (2001)
	32	CTAAATCAGAGCTACAAAGTG	
vatC	33	TGGCAAAATCAGCAAGG	Werner *et al.* (2001)
	34	TCGTCTCTATCTCTAGGTCC	
vgaA	35	AGTGGTGGTGAAGTAACACG	Werner *et al.* (2001)
	36	CTTGTCTCCTCCGCGAATAC	
vgaB	37	TCTCTCAATTAGAAGAACC	Werner *et al.* (2001)
	38	TTATCTATTCGTGTTTCC	
aac(6′)–aph(2″)	39	TTGGGAAGATGAAGTTTTTAGA	Martineau *et al.* (2000)
	40	CCTTTACTCCAATAATTTGGCT	

*The GenBank accession numbers for the *atl* and *pls* genes are D17366 and AF115379, respectively.

(2000). The *S. aureus* ATCC 29213, ATCC 25923, ATCC 43300 and RN4220 strains were used as references. The obtained PFGE profiles were analysed using 1D image analysis software (Kodak) and compared directly. For every pair, the Dice coefficient (DC) was calculated (DC=number of shared electrophoretic bands $\times 2 \times 100$/ overall number of electrophoretic bands in the two samples). Strains with a DC $\geqslant$80 % were considered to be strictly related, whilst those with a 60 % $\leqslant$DC $\leqslant$80 % were classified as related, and subsequent nomenclature was assigned according to the rules reported by Ripa *et al.* (2001).

RESULTS AND DISCUSSION

Antibiotic resistance

The results of the antibiotic resistance analyses are summarized in Table 2. Of the 37 strains, 89.2 % were resistant to PEN, whilst 43.2 % were resistant to OXA and were classified as MRSA. Nearly half of the strains were resistant to both ERY and CLI, and 88.2 % of this latter

Table 2. Phenotypes and genotypes of antibiotic resistance and biofilm formation

Strain	Resistance																			Biofilm				
	Phenotype										Gene									Gene				Mean A_{540}
	PEN	OXA	TET	ERY	CLI	TEL	GEN	CIP	QD	RIF	*blaZ*	*mecA*	*ermA*	*ermB*	*ermC*	*msrA*	*tetK*	*tetM*	*aac(6′)–aph(2″)*	*icaA*	*icaD*	*pls*	*atl*	
SA019	R	R	R	R	R	R	R	R	R	R	+	+	+	−	−	−	+	+	+	+	+	−	+	1.675
SA032	R	S	S	S	S	S	S	S	S	R	+	−	−	−	−	−	−	−	−	+	+	−	+	0.573
SA035	R	R	S	R	R	R	R	R	S	S	+	+	+	−	−	−	−	−	+	+	+	+	+	0.682
SA036	R	S	S	S	S	S	S	S	S	S	+	−	−	−	−	−	−	−	−	+	+	−	+	0.515
SA037	R	S	S	S	S	S	S	S	S	S	+	−	−	−	−	−	−	−	−	+	+	−	+	0.413
SA038	R	S	S	S	S	S	S	S	S	R	+	−	−	−	−	−	−	−	−	+	+	−	+	0.631
SA040	R	R	S	R	R	R	R	R	S	R	+	+	+	−	+	−	−	−	+	+	+	−	+	0.948
SA041	R	S	S	S	S	S	S	S	S	S	+	−	−	−	−	−	−	−	−	−	−	−	−	0.511
SA042	R	R	S	R	R	R	R	R	S	S	+	+	+	−	−	−	−	−	+	+	+	+	+	0.687
SA043	R	R	S	R	R	R	R	R	S	S	+	+	+	−	−	−	−	−	+	+	+	+	+	0.525
SA047	R	R	S	R	R	R	R	R	S	R	+	+	+	−	−	−	−	−	+	+	+	+	+	1.152
SA053	R	R	S	R	R	R	R	R	S	R	+	+	+	−	+	−	−	−	−	−	−	−	−	0.568
SA057	R	R	S	R	R	R	S	S	S	S	+	+	−	−	+	−	−	−	−	+	+	−	+	0.088
SA060	R	S	S	R	R	R	S	S	S	S	+	−	−	−	+	−	−	−	−	+	+	−	+	1.074
SA063	R	S	S	S	S	S	S	S	S	S	+	−	−	−	−	−	−	−	−	+	+	−	+	1.088
SA064	R	S	S	R	D^+	S	S	S	S	S	+	−	−	−	+	−	−	−	−	+	+	−	−	5.391
SA070	R	R	S	R	R	R	R	R	S	S	+	+	−	+	−	−	−	−	+	+	+	+	+	1.281
SA075	R	R	S	R	R	R	R	R	S	S	+	+	+	−	+	−	−	−	−	+	+	+	+	0.494
SA076	R	S	S	S	S	S	S	S	S	R	+	−	−	−	−	−	−	−	−	+	+	−	+	0.369
SA088	R	S	R	S	S	S	S	S	S	S	+	−	−	−	−	−	+	−	−	+	+	−	+	0.518
SA093	R	S	S	R	D^+	S	R	S	S	S	+	−	−	−	+	−	−	−	+	+	+	−	+	0.174
SA094	R	S	S	S	S	S	S	S	S	S	+	−	−	−	−	−	−	−	−	+	+	−	+	0.289
SA097	R	R	S	R	R	R	R	R	S	S	+	+	+	−	−	−	−	−	+	+	+	−	+	0.287
SA098	R	R	S	R	R	R	R	R	S	S	+	+	+	−	−	−	−	−	+	+	+	−	+	0.308
SA101	R	R	S	R	R	R	R	R	S	S	+	+	+	−	−	−	−	−	+	+	+	+	+	0.323
SA102	R	S	R	S	S	S	S	S	S	S	+	−	−	−	−	−	+	−	+	+	+	+	+	0.410
SA105	S	S	I	S	S	S	S	S	S	S	−	−	−	−	−	−	−	−	−	+	+	−	+	0.491
SA113	R	S	S	S	S	S	S	S	S	S	+	−	−	−	−	−	−	−	−	+	+	−	+	0.332
SA114	R	S	S	S	S	S	S	S	S	S	+	−	−	−	−	−	−	−	−	+	+	−	+	0.462
SA116	R	R	S	R	R	R	R	R	S	R	+	+	+	−	−	−	−	−	+	+	+	+	+	0.001
SA123	S	S	S	S	S	S	S	S	S	S	−	−	−	−	−	−	−	−	−	+	+	−	+	1.087
SA125	R	S	S	S	S	S	S	S	S	S	+	−	−	−	−	−	−	−	−	+	+	−	+	1.563
SA132	R	R	S	S	S	S	S	S	S	S	+	−	−	−	−	−	−	−	−	+	+	−	+	0.619
SA134	S	S	R	I	S	S	S	S	S	S	−	−	+	−	−	−	−	+	−	+	+	−	+	0.496
SA138	R	S	S	S	S	S	S	S	S	S	+	−	−	−	−	−	−	−	−	+	+	−	+	0.338
SA143	S	S	S	S	S	S	S	S	S	S	−	−	−	−	−	−	−	−	−	+	+	−	+	0.411
SA146	R	R	S	S	S	S	S	S	S	S	+	−	−	−	−	−	−	−	−	+	+	−	+	0.001

R, Resistant; S, susceptible; I, borderline resistance; D^+, inducibly resistant to CLI; +, positive in specific PCR; −, negative in specific PCR.

subgroup were not susceptible to TEL. Importantly, the two ERY/CLI-resistant and ketolide-susceptible strains showed an inducibly D^+ resistance pattern towards CLI, with a D-shaped zone of inhibition around the CLI disc (Steward *et al.*, 2005). One isolate out of thirty-seven was resistant to QD (strain SA019). TET was inactive towards 10.8 % of the isolates and partially active against one (strain SA105). Resistance towards members of the aminoglycosides (GEN), quinolones (CIP) and rifamycines (RIF) was recorded in 37.8, 35.1 and 21.6 % of the isolates, respectively. All isolates were susceptible to LZD (MIC_{50}=1.0 mg l^{-1}; MIC_{90}=2.0 mg l^{-1}) and vancomycin (MIC_{50}=0.5 mg l^{-1}; MIC_{90}=1.0 mg l^{-1}).

Subsequent PCR analysis of the genetic determinants of resistance was performed and related to the respective phenotypic pattern. As reported in Table 2, the population presented a full correlation between the presence of the *blaZ* genetic trait and resistance to PEN, whilst 2/16 MRSA isolates were *mecA* negative. The presence of specific resistance genes among the ERY-resistant group was also evaluated. All isolates were negative for the *msrA* gene, which encodes the staphylococcal ERY efflux system. Among ERY resistance methylase genes, the most prevalent was *ermA*, which was detected alone in half of the *erm*-positive subpopulation, whilst *ermB* was recorded in only one isolate. *ermA/ermC* association occurred in three isolates, whilst the *ermC* gene alone was detectable in four. All *ermA* and *ermA/ermC* genotypes were associated with the constitutive macrolide, lincosamide and streptogramin B phenotype (resistance to both ERY and CLI), except for isolate SA134, which had intermediate resistance and was susceptible to the macrolide and the lincosamide. In the group that was positive for *ermC* alone (n=4), half of the isolates showed a constitutive phenotype, whilst the other half were ERY-resistant and CLI-susceptible, expressing a D^+ phenotype (D-shaped zone of inhibition around the CLI disc).

tetK, tetM, tetL and *tetO* were evaluated to determine the TET resistance genotypic distribution. None of the tested isolates were positive for *tetL* or *tetO*. Two TET-resistant isolates (SA088 and SA102) carried *tetK*, whilst SA134 was positive for *tetM*. SA019 contained both *tetK* and *tetM*. The intermediate resistance phenotype did not harbour any Tet determinants. All GEN-resistant isolates and the susceptible SA102 isolate harboured *aac(6′)–aph(2″)*. QD-resistant SA019 was positive for *ermA* and *vgaA*.

This analysis of 37 *S. aureus* isolates from CVC-related infections confirmed the well-established multiresistant character of staphylococci in the hospital setting. We found that PEN, OXA, GEN and ERY co-resistance was very common and its genetic basis could be defined with the exception of a GEN-susceptible isolate containing the *aac(6′)–aph(2″)* gene and two MRSAs that were negative for the *mecA* gene. The latter occurrence has been observed previously in *S. aureus* and may be due, at least in part, to β-lactamase overexpression (Martineau *et al.*, 2000). SA019 was the single isolate that was resistant to all tested antibiotics, including QD. Its resistance to streptogramins was due to a methylase gene (*ermA*) and a specific gene encoding an ATP-binding protein (*vgaA*).

Only two MSSA were susceptible to all tested antibiotics. In general, MSSA did not show an extended multiresistance. For instance, 10/21 (47.6 %) MSSA were resistant only to one antibiotic (i.e. PEN or ERY), and 6/21 (28.6 %) were resistant to two (i.e. PEN/RIF, PEN/TET or TET/ERY).

We found a significant correlation between OXA resistance and resistance to ERY, CLI, TEL, GEN and CIP. This evidence is in line with previous reports and confirms the generally held idea that MRSA is resistant to several drugs and other toxic elements (e.g. cadmium) (Oliveira & Lencastre, 2002). In particular, CIP is a fluoroquinolone, which is among the most commonly prescribed classes of antimicrobial drugs in both the hospital and the community. By the early 1990s, many MRSA isolates from clinical specimens were found to be resistant to CIP. Moreover, several recent investigations have offered preliminary evidence suggesting that fluoroquinolones themselves may actually predispose patients to infection with or carriage of MRSA (Crowcroft *et al.*, 1999; Harnett *et al.*, 1991).

Biofilm formation

The presence of the *icaA* and *icaD* genes was assessed by PCR. One major group was observed (Table 2). Of the 37 clinical isolates that were analysed, 94.6 % contained both *icaA* and *icaD*. These genes were not found in two isolates. To obtain a semi-quantitative estimate of biofilm formation level, a crystal violet staining methodology was used. This method measures the amount of crystal violet stain retained by cultured bacterial cells adhering to the bottom of microtitre plate wells after repetitive cycles of washing. The mean intra-plate coefficient of variation was calculated by including an in-plate control strain; this variability was below 15 % (90 % degree of confidence). The mean inter-plate coefficient of variation for each isolate was below 20 % (90 % degree of confidence). All but three isolates presented a mean A_{540} value above 0.12, which is the lower limit for a biofilm-forming strain (Christensen *et al.*, 1985). In the *icaAD*-positive (*icaAD*$^+$) group, the mean A_{540} value was 0.734 (SD=0.906). Among the *icaAD*$^+$ isolates, SA057, SA116 and SA146 were the only biofilm-negative samples.

Two isolates were found to be *icaAD*-negative (*icaAD*$^-$). The mean A_{540} value of these isolates was 0.540 (SD=0.040 units).

Using PCR, we screened our population for the presence of both *pls* (an anti-adhesive factor) and *atl* (a factor involved in initial adherence) genes, and evaluated their individual and possible synergistic contributions to the formation of biofilms in the context of an *icaAD*$^+$ or *icaAD*$^-$ background. Their presence did not correlate significantly

with biofilm-forming capacity or the inability of the isolates to adhere to plastic surfaces.

Phenotypic and genotypic analyses of the ability of isolates to form biofilms *in vitro* revealed that the isolates achieved a good general level of growth on polystyrene surfaces. This finding is consistent with the site of isolation (i.e. catheters). However, a high degree of variability in biofilm formation capacity was recorded among isolates possessing the *icaAD* operon, ranging from strong biofilm producers to those that could not adhere to the plastic surface of the microtitre wells. In contrast, *icaAD*$^-$ isolates were able to produce a certain level of biofilm, well above that measured for at least half of the *icaAD*$^+$ isolates. The *ica* operon is considered to be one of the main genetic determinants of the accumulation phase during biofilm formation and its detection has been suggested as a tool for discriminating invasive from contaminating strains in clinical specimens (Arciola *et al.*, 2002; Vandecasteele *et al.*, 2003). Our observations showed, however, that *icaAD*$^+$, weak biofilm-producing *S. aureus* can be isolated from CVCs. The same conclusion has also been reached by other authors, supporting the general statement that the *ica* operon is not suitable as a discriminatory test for the capacity of invasiveness, at least in the case of *S. epidermidis* (Rohde *et al.*, 2004). On the one hand, isolates lacking the *ica* operon are able to sustain growth as a biofilm; however, some of the *ica* operon-positive isolates produce an equivalent or smaller amount of biofilm than *ica* operon-negative isolates. This finding is rather novel in the specific case of *S. aureus* isolated from CVCs, but is not surprising, as it has already been reported for coagulase-negative and coagulase-positive staphylococci (Cafiso *et al.*, 2004; de Silva *et al.*, 2002). Moreover, the SD calculated for the values of biofilm mass formed by *icaAD*$^+$ group is high. This is an indication of great variability and, at least in terms of biofilm mass production, seems to confirm that the presence of genes encoding the polysaccharide intercellular adhesin does not constitute an absolute determinant of biofilm-formation ability.

Genetic relatedness and SCC*mec* element analysis

Molecular analysis of *Sma*I-digested DNA resulted in 31 distinct PFGE types (Table 3), of which 45.2 % were recorded in only one isolate (one-strain type, ost). The remaining patterns fell into six type clusters (A–F). As

Table 3. Classification of the *S. aureus* strains under study based on genotypic traits related to meticillin resistance and PFGE

PFGE			SCC*mec* typing and *ccr* allotyping										
Type	**Subtype**	**No. of isolates**	***mecA***	***cif2***	***dcs***	***pubd***	***kdp***	***mecI***	***pt181***	***rif4***	***rif5***	**SCC*mec* type (no. of isolates)**	***ccr* type**
A	A1	4	−	ND	ND	ND	ND	ND	ND	ND	ND	−	−
	A2	1	−	ND	ND	ND	ND	ND	ND	ND	ND	−	−
	A3	1	−	ND	ND	ND	ND	ND	ND	ND	ND	−	−
B	B1	2	+	+	+	−	−	−	−	−	−	I	1
	B2	1	+	+	+	−	−	−	−	−	−	I	1
	B3	1	+	+	+	−	−	−	−	−	−	I	1
	B4	1	+	+	+	−	−	−	−	−	−	I	1
	B5	1	+	+	+	−	−	−	−	−	−	I	1
C	C1	2	−	ND	ND	ND	ND	ND	ND	ND	ND	−	−
	C2	1	−	ND	ND	ND	ND	ND	ND	ND	ND	−	−
	C3	1	−	ND	ND	ND	ND	ND	ND	ND	ND	−	−
D	D1	1	+	−	+	−	−	+	−	−	−	UT	−
	D2	1	−	ND	ND	ND	ND	ND	ND	ND	ND	−	−
	D3	1	−	ND	ND	ND	ND	ND	ND	ND	ND	−	−
E	E1	2	+	+	−	−	−	−	+	−	−	UT	1−2
			+	+	−	−	−	−	−	−	−	UT	1−2
F	F1	1	−	ND	ND	ND	ND	ND	ND	ND	ND	−	−
	F2	1	−	ND	ND	ND	ND	ND	ND	ND	ND	−	−
ost*	−	14	− (*n*=9)	ND	ND	ND	ND	ND	ND	ND	ND	−	−
			+ (*n*=5)	+	+	−	−	−	−	−	−	I (*n*=1)	1
				+	+	−	−	−	−	−	−	I (*n*=2)†	1
				+	+	+	−	+	−	−	−	UT (*n*=1)	1−3
				−	−	−	−	+	−	+	+	IIIA (*n*=1)	3

ND, Not determined; UT, unknown type.

*ost, One-strain type (PFGE type represented by a single strain).

†These two strains were negative for the amplification of *pls*.

expected, PFGE analysis closely correlated with the presence of *mecA* and meticillin resistance (Table 3). In just one PFGE class (type cluster D), both meticillin-susceptible (*mecA*-negative) and -resistant (*mecA*-positive) isolates were present. Type cluster B was related to archaic clones (COL and UK13136 strains), whilst the PFGE profile of the other strains did not match any of the pandemic clones used as references. Significant relatedness between PFGE patterns and other resistance phenotypes/genotypes was not observed.

To define SCC*mec* types, we applied a multiplex PCR assay designed by Oliveira *et al.* (2002) and further confirmed the assessment by *ccr* allotyping. Among the 14 *mecA*-positive strains, nine belonged to SCC*mec* type I-*ccrAB1*. Among the latter group, seven strains clustered in PFGE type B (Table 3). Strain SA019 was classified as SCC*mec* type IIIA-*ccrAB3*, but its PFGE profile did not match any of the IIIA-positive Brazilian pandemic clonal type strains used as references, namely HU25 and HSJ216. The remaining four *mecA*-positive strains were found to possess a SCC*mec* element that was only partially or not at all related to any of the previously described types; these strains were therefore classified as unknown type (Table 3). Strains SA097 and SA098 showed PCR amplification profiles consistent with type I, but with positive amplification of the *cif*2 region and negative amplification of the coding region for the *pls* gene (Table 2). This variation was additionally confirmed by the impossibility of clustering the PFGE profile of these strains with those of the type I archaic clones. Nevertheless, these strains conserved a type I basic structure as revealed by the positive amplification of the *ccrAB1* complex. Strain SA075 was positive for all of the elements that characterize the SCC*mec* type IA with the additional amplification of the *mecI* portion. The *ccr* analysis confirmed the relationship with type I, but also indicated a relationship with the type III *ccr* (Table 3). Analysis of SA040, SA053 and SA057 did not result in classification of the SCC*mec* elements harboured by these strains. SA040 was positive for *cif*2, but not for the *dcs* region that is characteristic of the type I SCC*mec*, and it contained both *ccrAB1* and *ccrAB2*. *cif*2 was associated with pT181, *ccrAB1* and *ccrAB2* in SA053, whilst SA057 would be compatible with a type IV SCC*mec* with the exception that it was positive for the *mecI* region. Moreover, in the latter strain, none of the tested *ccr* complexes was amplified, even when the universal pair of primers, which amplifies all types of known *ccr* complexes, was used (Table 3).

The striking finding of this study is the degree of genetic variation within SCC*mec* in MRSA isolated from catheter-associated infections. At the genomic level, this evidence was also confirmed by *Sma*I macrorestriction profiling. Two strains appeared to be variants of the archaic clone MRSA-I. They lacked a portion of the L-C region mapping 5′ of the type I SCC*mec*. Shore *et al.* (2005) described a similar SCC*mec* I type element in nine strains isolated in Ireland. This region contains *pls*, which is not thought to be an important part of SCC*mec* (Ito *et al.*, 2004), but it has been implicated in biofilm formation in staphylococci (Savolainen *et al.*, 2001). However, the adhesion properties of these SCC*mec*I-*pls*$^{-}$ strains did not differ significantly from either the parental SCC*mec*I or from the other MRSA and MSSA strains. One strain showed a type IA variant that was positive for amplification of the *mecI* region and the *ccrAB* complex of both types I and III. This observation could be explained either by a genetic rearrangement that occurred between genetic cassettes belonging to the two different classes or by the transfer of a new SCC*mec* element variant from another species. Lastly, three strains presented genetic organizations of SCC*mec* that could not be related to any of the known variants of this element, confirming that there have been major changes in the dominant clonal types in this group of strains from catheter-related infections in hospitalized patients. This genetic individuality was also confirmed by PFGE typing and supports the great flexibility of MRSA to evolve in addition to its general diffusion as pandemic clones. To the best of our knowledge, this is the first study reporting such variability in a set of strains isolated from CVCs. In this context, the high frequency of isolation of atypical MRSA from device-related sources of infection raises concerns over the possible importance of growth on surfaces for the selection of new variants. This hypothesis is supported by the observation reported by Prunier *et al.* (2003) that some bacterial populations growing as biofilms present a high proportion of hypermutable strains, and by the fact that the exchange of genetic material between bacteria, and consequent genetic rearrangement by recombination, is greatly favoured in the particular environment created by a biofilm (Molin & Tolker-Nielsen, 2003).

ACKNOWLEDGEMENTS

We are grateful to Dr D. Mack for *S. epidermidis* strains 1457 and 1457-M11, and to Dr H. de Lencastre and A. Tomasz for all *S. aureus* reference pandemic strains. This work was supported by a grant from the Italian Ministry of University and Scientific Research, PRIN 2005 – 2005061134_002 (to M. P.).

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