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Detection of Staphylococcal Cassette Chromosome *mec* Type XI Carrying Highly Divergent *mecA*, *mecI*, *mecR1*, *blaZ*, and *ccr* Genes in Human Clinical Isolates of Clonal Complex 130 Methicillin-Resistant *Staphylococcus aureus*▽†

Anna C. Shore,[1] Emily C. Deasy,[1] Peter Slickers,[2] Grainne Brennan,[3] Brian O'Connell,[3,4] Stefan Monecke,[5] Ralf Ehricht,[2] and David C. Coleman[1]*

Microbiology Research Unit, Dublin Dental University Hospital, University of Dublin, Trinity College Dublin, Dublin, Ireland[1]*; Alere Technologies GmbH, Jena, Germany*[2]*; National MRSA Reference Laboratory, St. James's Hospital, James's St., Dublin 8, Ireland*[3]*; Department of Clinical Microbiology, University of Dublin, Trinity College Dublin, St. James's Hospital, James's St., Dublin 8, Ireland*[4]*; and Institute for Medical Microbiology and Hygiene, Faculty of Medicine Carl Gustav Carus, Technical University of Dresden, Dresden, Germany*[5]

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Methicillin resistance in staphylococci is mediated by penicillin binding protein 2a (PBP 2a), encoded by *mecA* on mobile staphylococcal cassette chromosome *mec* (SCC*mec*) elements. In this study, two clonal complex 130 (CC130) methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from patients in Irish hospitals were identified that were phenotypically PBP 2a positive but lacked *mecA* by conventional PCR and by DNA microarray screening. The isolates were identified as methicillin-susceptible *S. aureus* using the GeneXpert real-time PCR assay. Whole-genome sequencing of one isolate (M10/0061) revealed a 30-kb SCC*mec* element encoding a class E *mec* complex with highly divergent *blaZ-mecA-mecR1-mecI*, a type 8 cassette chromosome recombinase (*ccr*) complex consisting of *ccrA1-ccrB3*, an arsenic resistance operon, and flanking direct repeats (DRs). The SCC*mec* element was almost identical to that of SCC*mec* type XI (SCC*mec* XI) identified by the Sanger Institute in sequence type 425 bovine MRSA strain LGA251 listed on the website of the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements. The open reading frames (ORFs) identified within SCC*mec* XI of M10/0061 exhibited 21 to 93% amino acid identity to ORFs in GenBank. A third DR was identified ca. 3 kb downstream of SCC*mec* XI, indicating the presence of a possible SCC remnant. SCC*mec* XI was also identified in the second CC130 MRSA isolate by PCR and sequencing. The CC130 MRSA isolates may be of animal origin as previously reported CC130 *S. aureus* strains were predominantly from bovine sources. The highly divergent nature of SCC*mec* XI relative to other SCC*mec* elements indicates that it may have originated in another taxon.

Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates are *S. aureus* isolates that exhibit phenotypic resistance to methicillin and related β-lactam antibiotics, including cefoxitin and oxacillin. The first report of MRSA was in England in 1961 (24), and since then MRSA has become a major nosocomial problem. Today MRSA is one of the most important antibiotic-resistant pathogens in hospitals and communities around the world. There is also an increasing awareness of the significance of MRSA in animals and of the zoonotic potential of MRSA (69).

The success of *S. aureus* is partly due to its ability to acquire mobile genetic elements encoding virulence and antimicrobial resistance genes from other *S. aureus* isolates and from coagulase-negative staphylococcal (CoNS) species (12, 17, 21, 33, 46, 57, 58, 66). One such group of mobile genetic elements are the staphylococcal cassette chromosome *mec* (SCC*mec*) elements, which harbor the methicillin resistance gene *mecA* (22). Existing MRSA clones have arisen following the acquisition and integration of SCC*mec* into a specific site (the integration site sequence [ISS]) within an open reading frame (ORF) in the *S. aureus* chromosome designated *orfX* (21, 22). Until recently, the function of *orfX* was unknown, but in recent months it has been annotated as a 23S rRNA methyltransferase (http://www.uniprot.org/uniprot/P0C1V0). SCC*mec* elements are characterized by the presence of flanking direct repeat (DR) sequences that are generated at both ends of the integrated SCC*mec* element (22). The *mecA* gene encodes the additional penicillin binding protein (PBP) 2a (in addition to PBP A to PBP D or PBP 1 to PBP 4) with a low affinity for β-lactam antibiotics, and the expression of *mecA* in MRSA allows cell wall synthesis to continue, despite inactivation of native PBPs by β-lactams (21, 48). The *mecA* gene forms part of the *mec* gene complex in SCC*mec* elements. Four classes of the *mec* gene complex (A to D), which differ in their genetic organization, have been described in the published literature (21, 26). Each has an intact copy of *mecA*, a copy of insertion sequence IS*431*, and, when present, complete or truncated *mec* regula-

* Corresponding author. Mailing address: Microbiology Research Unit, Division of Oral Biosciences, School of Dental Science, Dublin Dental University Hospital, University of Dublin, Trinity College Dublin, Dublin 2, Ireland. Phone: 353 1 612 7276. Fax: 353 1 612 7295. E-mail: david.coleman@dental.tcd.ie.

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tory genes *mecI* and *mecR1*. The cassette chromosome recombinase (*ccr*) genes form another essential component of SCC*mec* that are involved in site-specific integration and excision of SCC*mec* (21, 27). To date, five *ccr* gene complexes (*ccrA1B1*, *ccrA2B2*, *ccrA3B3*, *ccrA4B4*, and *ccrC*) and eight SCC*mec* types (I to VIII) have been described in MRSA in the published literature, with the latter characterized by the presence of different combinations of *ccr* and *mec* complex genes and with subtypes defined by variations in the junkyard (J) regions (7, 11, 21–23, 31, 45, 56, 75). Furthermore, some additional *mec*, *ccr*, and SCC*mec* types that have not yet been described in the published literature are listed on the SCC*mec* website curated by the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC; http://www.sccmec.org/Pages/SCC_TypesEN.html). Because of the extensive diversity that has been observed in the genetic organization of SCC*mec* elements, the IWG-SCC was established to standardize criteria for the classification of SCC*mec* elements. Advice on appropriate nomenclature for novel SCC*mec* types and subtypes is available from the IWG-SCC, and it is recommended that the entire nucleotide sequence of all novel SCC*mec* elements be determined prior to SCC*mec* type designation (21).

SCC*mec* elements are not exclusive to MRSA and have also been reported in several CoNS species, many of which form part of the normal flora of healthy human and animal skin and mucous membranes and are frequently associated with opportunistic human and veterinary infections (9, 15, 18, 53, 62, 66, 70, 76). The presence of very similar SCC*mec* elements in MRSA and CoNS strongly suggests horizontal transfer of SCC*mec* (6, 8, 10, 17, 18, 61, 70). While the mechanism(s) and the exact route of transfer of SCC*mec* are presently unknown, the higher frequency and diversity of SCC*mec* elements in CoNS suggest that CoNS are potentially a reservoir of SCC*mec* elements that may facilitate the emergence of new MRSA clones (15, 16). In addition, several studies have identified *mecA* gene homologs with and without *mec* complex genes, as well as SCC elements without *mecA* but with *ccr* genes, as individual genetic components in CoNS and in the closely related species *Macrococcus caseolyticus* (40, 55, 65, 66, 73, 74). Thus, some researchers have speculated that such genetic elements may be the evolutionary precursors of SCC*mec* in CoNS and MRSA (66, 72).

In Ireland, MRSA has been endemic in hospitals since the 1980s, and different strains have predominated at different time periods (56). For the last decade, sequence type 22-MRSA-SCC*mec* type IV (ST22-MRSA-IV) isolates have dominated, and since 2003 they have accounted for 80% of MRSA isolates recovered from patients in Irish hospitals (51, 59). In line with trends worldwide, MRSA isolates harboring the Panton-Valentine leukocidin gene have emerged as community-acquired pathogens in Ireland and MRSA has also been reported among animals (1, 2, 32, 41, 52).

In the present study, we report on the identification of human clinical MRSA isolates from Ireland belonging to clonal complex 130 (CC130) that are detected as PBP 2a positive using two different latex agglutination assays but lack *mecA*, as detected by conventional *mecA* PCR. These MRSA isolates were detected as methicillin-susceptible *S. aureus* (MSSA) using the GeneXpert real-time PCR assay, which targets SCC*mec* and *mecA*, as well as the *S. aureus* protein A gene *spa* and the ISS, for the rapid detection of MRSA in clinical specimens (71). A comprehensive analysis of these isolates resulted in the identification of a novel SCC*mec* element with highly divergent *mecA*, *mecI*, *mecR1*, *blaZ*, and *ccr* genes.

MATERIALS AND METHODS

***Staphylococcus aureus* isolates investigated.** Two clinical MRSA isolates (M10/0148 and M10/0061) were investigated in the present study. M10/0148 was isolated from a 64-year-old female inpatient in an acute care hospital in Dublin, Ireland, in February 2010, and M10/0061 was isolated from an 85-year-old male inpatient in a regional hospital in the southeast of Ireland in May 2010. There was no obvious epidemiological connection between these patients, and the two hospitals are approximately 160 kilometers apart. There was no information available indicating that the patients had had any contact with animal husbandry, domestic animals, raw meat, or other potential sources of zoonotic bacteria. Isolates were identified as *S. aureus* by detection of staphylocoagulase production (50), were inoculated onto Protect beads (Technical Service Consultants Limited, Heywood, United Kingdom), and stored at −70°C prior to subsequent detailed analysis.

A MSSA isolate (21250_V454) recovered from a brown rat (*Rattus norvegicus*) in Germany (courtesy of Sebastian Günther, Berlin University) was also included in the study for comparison since it was found to belong to the same clonal lineage and yielded DNA microarray hybridization patterns (see below) similar to those of the two clinical isolates.

Investigation of resistance to antimicrobial agents. Screening for oxacillin/methicillin resistance was performed by culture of isolates on cefoxitin-containing ChromID MRSA selective agar (bioMérieux, Nuertlingen, Germany). The isolates were tested for methicillin resistance using 30-μg cefoxitin disks (Oxoid, Basingstoke, Hampshire, United Kingdom) on BBL Mueller-Hinton II agar (Becton Dickinson and Company, Sparks, MD) using the Clinical and Laboratory Standards Institute (CLSI) methodology (13). The MRSA isolates were further tested against oxacillin (1-μg and 5-μg disks; Oxoid) on Columbia blood agar (CBA; Oxoid) at 30°C. Oxacillin MICs were determined using the Etest system (bioMérieux) according to the manufacturer's instructions, and oxacillin MIC determinations were repeated using induced colonies that had been exposed to oxacillin. Additionally, susceptibility tests for the MRSA isolates were performed using the Vitek-2 system according to the manufacturer's instructions (Gram-positive bacteria susceptibility test card; bioMérieux). Both the MSSA isolate and the MRSA isolates were tested for PBP 2a production using a Mastalex MRSA kit (Mast Diagnostics, Bootle, United Kingdom), an MRSA-Screen latex agglutination assay (Innogenetics, Gent, Belgium), and a Clearview Exact PBP 2a kit (Alere, Cologne, Germany). All isolates were also tested for the presence of *mecA* by PCR using previously described primers (44) and novel primers to amplify the entire *mecA* gene (Table 1), with MRSA strains N315 (29) and ATCC 43300 as positive controls and MSSA strains MSSA476 (19, 29) and ATCC 25923 as negative controls. The MRSA isolates underwent antibiogram-resistogram (AR) typing as described previously to determine their resistance to a panel of 23 antimicrobial agents (52). The AR typing panel consists of the following antimicrobial agents: amikacin, ampicillin, cadmium acetate, chloramphenicol, ciprofloxacin, erythromycin, ethidium bromide, fusidic acid, gentamicin, kanamycin, lincomycin, mercuric chloride, mupirocin, neomycin, phenyl mercuric acetate, rifampin, spectinomycin, streptomycin, sulfonamide, tetracycline, tobramycin, trimethoprim, and vancomycin (52). The MRSA isolates were tested for susceptibility to arsenicals using 100-, 250-, and 500-μg sodium arsenite disks and 250-, 500-, and 1,000-μg sodium arsenate disks and the CLSI methodology (13). The arsenic-resistant *S. aureus* reference strain WGB8404 (43) and the arsenic-susceptible *S. aureus* reference strain RN4220 (28) were used as controls. The MRSA isolates were also tested for susceptibility to sodium arsenite by broth microdilution. For these experiments, the arsenic-susceptible *S. aureus* strain NCTC8325 (42) and the arsenic-resistant strain WGB8404 were used as controls. Briefly, doubling dilutions of sodium arsenite (range, 10 to 10,000 μg/ml) were prepared in nutrient broth (Oxoid) supplemented with 2% (wt/vol) glucose. Inocula were prepared from cultures grown on CBA and adjusted to a density corresponding to a 0.5 McFarland standard (bioMérieux), and 20 μl was added to the wells of 96-well microtiter plates containing 80 μl of the respective sodium arsenite dilutions. Following overnight incubation at 37°C, plates were examined visually for growth.

Investigation of isolates using the GeneXpert real-time PCR assay for the rapid detection of MRSA in clinical specimens. The MRSA isolates M10/0148 and M10/0061 were both tested using the Xpert MRSA/SA SSTI assay on a

TABLE 1. Novel PCR primers used in the study

Gene or region amplified	Primer pair	Nucleotide sequence (5′–3′)	Nucleotide coordinates
mecA	mecA_fw	ACCTTCTACACCTCCATATCAC	24320–24341[a]
	mecA_rv	CGTTACGGATTGCTTCACTG	26452–26433[a]
pbp1	Pbp1_fw	CGATAATGTAAAGGTAGTGCGTAG	828245–828222[b]
	Pbp1_rv	CCCTTATTTAATGTAGCCATTCTTG	825960–825984[b]
pbp2	Pbp2_fw	GATGAAAGTGAGGACCGCG	477649–477631[b]
	Pbp2_rv	ATAACTGAGGATTTTATGTTGAGTGG	475419–475444[b]
pbp3	Pbp3_fw	GAAAGACTTTGAATAGAGGTAGGTAG	332226–332251[b]
	Pbp3_rv	TGCGCTACACAATCGTCAG	334356–334338[b]
pbp4	Pbp4_fw	TTGGAAAAGGGAAGATTAACGC	308863–308884[c]
	Pbp4_rv	GCAACTTGTCCGTTTTTAGTATG	310208–310186[c]
$ccrA1B3_{M10/0061}$	$ccrB3_{M10/0061}$ F1	CTACTTGAAGTTATCCAATC	10125–10144[d]
	$ccrA1_{M10/0061}$ R1	CACATTACTCGCTGATTTAG	13427–13408[d]
$mecA_{M10/0061}$	$mecA_{M10/0061}$ F1	CCAGATATAGTAGCATTATA	1799–1818[d]
	$mecA_{M10/0061}$ R1	AAAGATGACGATATTGAG	3672–3655[d]
orfX-$mecI_{M10/0061}$	orfX F1	GTGTTAATTGAGCAAGTGTA	418–437[d]
	$mecI_{M10/0061}$ R2	GCATTCTTACATCCGTTTTG	6070–6051[d]
$mecI_{M10/0061}$-$ccrA1_{M10/0061}$	$mecI_{M10/0061}$ F1	CTATGATATATCAGCGTCAG	56041–56023[d]
	$ccrA1_{M10/0061}$ R1	ACAAGCTCAAGCGATACGGT	12563–12544[d]
$ccrA1_{M10/0061}$-$ORF\_119_{M10/0061}$	$ccrA1_{M10/0061}$ F1	AATAACGTAATGTGCGGTGC	12421–12440[d]
	$ORF\_119_{M10/0061}$ R1	ACTTAAATCAGGCTTATATCC	20622–20602[d]
$ORF\_119_{M10/0061}$-$arsC_{M10/0061}$	$ORF\_119_{M10/0061}$ F1	TCTAGTATCTGAAAGGTC	20475–20492[d]
	$arsC_{M10/0061}$ R1	AACCATACGTCAGACTTGA	27963–27945[d]
$arsC_{M10/0061}$-downstream of DR3	$arsC_{M10/0061}$ F1	GACCACTCTTTACCTGCT	27809–27826[d]
	tnp1S2 R1	AGATCATGGAAAACCGATCA	34397–34378[d]

[a] Nucleotide coordinates based on the nucleotide sequence of SCC*mec* III (GenBank accession number AB037671.1).
[b] Nucleotide coordinates based on the nucleotide sequence of *S. aureus* strain MN8 contig 215 (GenBank accession number ACJA02000004.1).
[c] Nucleotide coordinates based on the nucleotide sequence of *S. aureus* strain MN8 contig 215 (GenBank accession number ACJA02000003.1).
[d] Nucleotide coordinates based on the nucleotide sequence of SCC*mec* XI of M10/0061 (GenBank accession number FR823292).

GeneXpert DX system (version 1.2) real-time PCR platform (Cepheid, Sunnyvale, CA), according to the manufacturer's instructions, to determine if the MRSA isolates would be detected as MRSA using this system, which targets SCC*mec*-associated and *spa* DNA sequences. The MSSA isolate 21250_V454 was also tested.

Total genomic DNA isolation. Genomic DNA was extracted from all isolates by enzymatic lysis using the buffers and solutions provided with the StaphyType DNA microarray kit (described below) and the Qiagen DNeasy kit (Crawley, West Sussex, United Kingdom) as described previously (35).

Molecular typing. The isolates underwent *spa* typing and multilocus sequence typing (MLST) as described previously (14, 60).

DNA microarray analysis. The isolates were subjected to DNA microarray analysis using the StaphyType kit (Alere Technologies GmbH), which detects 334 *S. aureus* gene sequences and alleles, including species-specific, antimicrobial resistance- and virulence-associated genes, and typing markers (35). The genes detected using the array include previously described SCC*mec*-associated gene sequences, including *mecA*, its regulatory genes *mecI* and *mecR1*, various allotypes of the *ccr* genes, and gene sequences previously identified in the J regions of various SCC*mec* elements. The DNA microarray procedures have been described previously and were performed according to the manufacturer's instructions (35). DNA microarray analysis of each isolate was performed on three separate occasions, and two different labeling approaches were employed. In one, amplification and labeling were directed by target-specific primers (35). In the second, the complete genome was labeled using randomized oligonucleotides instead of the site-specific labeling as in the first method (37, 39). The latter protocol proved to be useful for the detection of mutated or divergent target genes, e.g., genes from strains that are not fully represented by the published *S. aureus* genome sequences due to the different stringency of the overall labeling and hybridization process.

PCR and sequencing of the genes encoding the native *S. aureus* PBPs 1 to 4. The *S. aureus* genes encoding PBPs 1 to 4 were amplified and sequenced using *Taq* polymerase (Genaxxon BioScience GmbH, Biberach, Germany), the primers PBP 1 to PBP 4 (Table 1), and the following conditions: 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 55°C for 20 s, and 72°C for 2 min, with a final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis in 1% (wt/vol) agarose gels, ethidium bromide staining, and visualization under UV light. Bands were extracted from agarose gels, and DNA was purified using a Qiaex II gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequencing was performed using the PCR primers and BigDye Terminator cycle sequencing kit (version 1.1; Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. Sequence analysis was performed using a 3130 genetic analyzer (Applied Biosystems).

Whole-genome sequencing of M10/0061. The whole genome of one MRSA isolate, M10/0061, was sequenced to investigate the location of the *ccr* genes identified in both isolates using the DNA microarray and to investigate the possible presence of a novel SCC*mec* element. High-throughput *de novo* sequencing was undertaken commercially by Geneservice (Source BioScience plc, Nottingham, United Kingdom) using an Illumina genome analyzer system (HiSeq 2000 platform; Essex, United Kingdom). The average coverage across the genome was 40 times. The reads were assembled into contigs using a Velvet *de novo* genome assembler (version 1.0.15; Illumina). Contigs were analyzed using the Artemis DNA sequence viewer and annotation tool (54) and BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (3). ORFs were predicted using prodigal (http://prodigal.ornl.gov/) (20), a gene prediction algorithm designed specifically for prokaryotic sequences. Prodigal was trained with four fully sequenced and annotated *S. aureus* genomes (GenBank accession numbers CP000046.1, BA000017.4, BX571856.1, and AJ938182.1). All predicted ORFs were analyzed against a local database of all staphylococcal sequences extracted from GenBank using the BLAST standalone software package (version 2.2.24; NCBI). The sequences of the ORFs were globally aligned with the best-matching sequences from the local database, and the positions of start and stop codons and potential ribosomal binding sites were checked for consistency. All ORFs were translated into protein sequences, and the protein sequences were analyzed against the NCBI nonredundant database of protein sequences with the BLAST program. Amino acid identities between two sequences were calculated from global sequence alignments. The sequences were aligned with the needle global alignment program from the EMBOSS software package (49). Relative identities were calculated from the number of identical amino acids in the alignment with respect to the length of the new sequence from SCC*mec* type XI (SCC*mec* XI). These overall amino acid identities were calculated for the best-matching BLAST sequence. In addition, for the *mec* complex genes, amino acid identities were calculated for additional sequences which represented different allotypes or paralogs of the gene concerned. Occurrences of the characteristic DRs of SCC*mec* were detected with the bl2seq program from the NCBI BLAST software package.

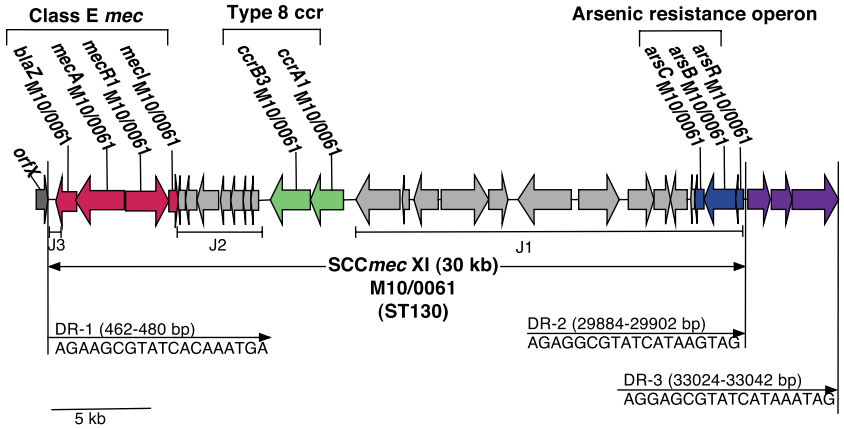

FIG. 1. Schematic diagram showing the genetic organization of the SCC*mec* element designated SCC*mec* XI and the adjacent 3-kb downstream region in the ST130 MRSA isolate M10/0061 (GenBank accession number FR823292). SCC*mec* XI is bordered by DR sequences DR-1 and DR-2, and the 3-kb downstream region is bordered by DR-2 and DR-3. The *mec* complex, *ccr* complex, and arsenic resistance operon genes are shown in red, green, and blue, respectively. The three ORFs identified in the adjacent 3 kb downstream region are shown in purple. A full list of ORFs encoded by the SCC*mec* element and adjacent region is provided in Table S1 in the supplemental material. The structure of the SCC*mec* element was determined by high-throughput whole-genome sequencing of M10/0061.

PCRs to confirm the presence of novel *mecA* and *ccr* allotypes and SCC*mec* element in M10/0148. Having determined the presence of novel allotypes of *ccr*, *mecA*, *blaZ*, and the *mec* regulatory genes *mecI* and *mecRI* in M10/0061 by analysis of its whole-genome sequence, primer pairs $ccrB3_{M10/0061}$ F1/$ccrA1_{M10/0061}$ R1 and $mecA_{M10/0061}$ F1/$mecA_{M10/0061}$ R1 were designed to investigate M10/0148 for the presence of $ccrA1B3_{M10/0061}$ and $mecA_{M10/0061}$, respectively (Table 1). In addition, primer pair orfX F1/$mecI_{M10/0061}$ R2 was used to amplify and sequence the complete *mec* gene complex in M10/0148, and four additional primer pairs were used to amplify the remainder of the novel SCC*mec* element (between DRs 1 and 2) and the 3-kb region identified between the second and third DRs in M10/0061 in M10/0148 (Table 1 and Fig. 1). DNA fragments were obtained by PCR amplification of chromosomal DNA using either GoTaq DNA polymerase (Promega Corporation, Madison, WI) for primers $ccrB3_{M10/0061}$ F1/$ccrA1_{M10/0061}$ R1 and $mecA_{M10/0061}$ F1/$mecA_{M10/0061}$ R1 or the Expand Long-Template PCR system (Roche Diagnostics Ltd., Lewes, East Sussex, United Kingdom) for all other primer pairs according to the manufacturers' instructions. The whole-genome-sequenced isolate M10/0061 harboring the novel *mecA* allotype and MRSA strain CA05 (31) harboring SCC*mec* IV and encoding the original *mecA* allotype were used as positive and negative controls, respectively. PCR products were visualized by agarose gel electrophoresis, gel red staining (Biotium, Hayward, CA), and visualization under UV light. PCR products were purified using a Genelute PCR cleanup kit (Sigma-Aldrich Chemical Co., Tallaght, Dublin, Ireland). DNA sequencing was performed commercially by Geneservice (Source Bioscience, St. James's Hospital, Dublin, Ireland). Sequence analysis was performed using the BLAST, BioNumerics (version 5.1; Applied Maths, Ghent, Belgium), and DNA Strider software packages.

Nucleotide sequence accession numbers. The nucleotide sequence of the SCC*mec* element (SCC*mec* XI) and the 3-kb DNA sequence identified adjacent to SCC*mec* XI were deposited in GenBank under the accession number FR823292. The nucleotide sequences of PBPs 1, 2, 3, and 4 identified in M10/0061 by whole-genome sequencing have been deposited in GenBank under the accession numbers FR823293, FR823294, FR823295, and FR823296, respectively.

RESULTS

Identification of two PBP 2a-positive clinical MRSA isolates that lack *mecA* by conventional *mecA* PCR. The *S. aureus* isolates M10/0061 and M10/0148 exhibited resistance to cefoxitin (30-μg cefoxitin disks) and oxacillin (1-μg and 5-μg disks), exhibited growth on ChromID MRSA selective agar, and were identified by the Vitek-2 system as MRSA. The two isolates exhibited oxacillin MICs of 1.0 mg/liter and 2.0 mg/liter, respectively, and for both isolates, these rose to 16 mg/liter when the colonies were induced (i.e., cultured in the presence of oxacillin) prior to oxacillin MIC determination. Both isolates were positive for production of PBP 2a using the Mastalex and Clearview latex agglutination assays but were PBP 2a negative using the Innogenetics PBP 2a assay. Furthermore, neither isolate yielded any amplicons following PCR using *mecA* primers, while the positive control MRSA strains N315 and ATCC 43300 yielded amplicons corresponding to the amplification of *mecA*. AR typing revealed that the isolates exhibited resistance to ampicillin only out of the 23 antimicrobials tested in the AR typing panel. Both MRSA isolates exhibited resistance to sodium arsenite and sodium arsenate.

The MSSA isolate did not grow in the presence of cefoxitin, was negative for the production of PBP 2a using the Mastalex, Innogenetics, and Clearview latex agglutination assays, and was *mecA* negative by both *mecA* PCR assays.

GeneXpert. The two MRSA isolates, M10/0061 and M10/0148, were identified as *S. aureus* but not as MRSA using the GeneXpert MRSA real-time PCR assay. Identical results were obtained for the rat MSSA isolate 21250_V454.

Molecular typing. The two MRSA isolates exhibited two different STs, ST130 and ST1764, following MLST analysis, with both belonging to CC130 (Table 2), respectively. ST1764 is a single-locus variant of ST130 that differs by only 1 nucleotide base within the *tpi* gene. The MSSA isolate was also assigned to CC130 (Table 2). Using *spa* typing, the MRSA isolates exhibited two closely related *spa* types (t843 and t373) (Table 2), which differ by the presence of two additional repeat units in t843. The MSSA isolate exhibited a novel but related *spa* type t8403 which differed from *spa* type t843 in the deletion of one repeat unit and from t373 in the presence of one additional repeat unit.

DNA microarray analysis. The DNA microarray results for the two MRSA isolates and the MSSA isolate are shown in Table 2. DNA microarray analysis confirmed the absence of *mecA* in the MSSA and MRSA isolates. No *mec* regulatory genes were detected. The MSSA and MRSA isolates yielded

TABLE 2. MLST, *spa* types, and DNA microarray hybridization profiles of MRSA isolates M10/0061 and M10/0148 and MSSA isolate 21250_V454

Gene class and gene(s)	DNA microarray[a] hybridization profile		
	M10/0061	M10/0148	21250_V454
Species markers *katA*, *coa*, *nuc*, *spa*	Pos	Pos	Pos
agr group III	Pos	Pos	Pos
SCC*mec*-associated markers			
mecA	Neg	Neg	Neg
Δ*mecR1*	Neg	Neg	Neg
mecR1-mecI	Neg	Neg	Neg
ccrA1	Neg	Neg	Neg
ccrB1	Variable	Variable	Neg
Q9XB68-*dcs*	Neg	Neg	Neg
ccrA2-ccrB2	Neg	Neg	Neg
kdp-SCC locus	Neg	Neg	Neg
ccrA3	Variable	Variable	Neg
ccrB3	Variable	Variable	Neg
ccrC, *ccrA4-ccrB4*	Neg	Neg	Neg
Antimicrobial resistance genes			
blaZ	Neg	Neg	Neg
blaI-blaR1	Neg	Neg	Neg
erm(A), *erm*(B)	Neg	Neg	Neg
erm(C)	Neg	Neg	Neg
lnu(A)	Neg	Neg	Neg
msr(A), *mph*(C)	Neg	Neg	Neg
aacA-aphD	Neg	Neg	Neg
aadD	Neg	Neg	Neg
aphA3-sat	Neg	Neg	Neg
dfrS1	Neg	Neg	Neg
far1	Neg	Neg	Neg
mupA	Neg	Neg	Neg
tet(K)	Neg	Neg	Neg
tet(M)	Neg	Neg	Neg
cat	Neg	Neg	Neg
cfr	Neg	Neg	Neg
fexA	Neg	Neg	Neg
fosB	Neg	Neg	Neg
tet efflux	Pos	Pos	Pos
Virulence-associated genes			
tst1	Neg	Neg	Neg
sea, *seb*, *see*, *seh*	Neg	Neg	Neg
sec-sel, *sed-sej-ser*	Neg	Neg	Neg
seg-sei-sem-sen-seo-seu	Neg	Neg	Neg
sek-seq	Neg	Neg	Neg
lukF-PV–lukS-PV	Neg	Neg	Neg
lukF-PV (P83), *lukM*	Neg	Neg	Pos
hla, hld	Pos	Pos	Pos
hlb	Pos	Pos	Pos
Untruncated *hlb*	Pos	Pos	Pos
sak-chp-scn	Neg	Neg	Neg
etA, etB, etD	Neg	Neg	Neg
edinA, edinB	Neg	Neg	Neg
edinB	Pos	Pos	Pos
arcA-arcB-arcC-arcD	Neg	Neg	Neg
Capsule type 8	Pos	Pos	Pos

[a] The StaphyType Kit (Alere Technologies) was used for DNA microarray analysis. The table shows only the clinically relevant markers, including exotoxins and resistance genes, and does not represent the complete hybridization profile of 334 probes on the array. Full data sets are available upon request. Isolate M10/0061 was CC130/ST130 and had the MLST allelic profile 6-57-45-2-7-58-52, *spa* type t843, and *spa* repeat succession 04-82-17-25-17-25-25-16-17. Isolate M10/0148 was CC130/ST1764 and had the MLST allelic profile 6-57-45-2-7-193-52, *spa* type t373, and *spa* repeat succession 04-17-25-17-25-16-17. Isolate 21250_V454 was CC130/ST2024 and had the MLST allelic profile 6-57-45-2-219-58-52, *spa* type t8403, and *spa* repeat succession 04-82-17-25-25-25-16-17. Pos, positive; Neg, negative; Variable, MRSA isolates M10/0061 and M10/0148 exhibited weak and/or variable signals for *ccrB1*, *ccrA3*, and *ccrB3* in the primer-directed protocol but yielded positive signals for *ccrB1* and *ccrB3* in the random protocol outlined in the Materials and Methods.

very similar DNA microarray signals, except that the MRSA isolates exhibited weak and/or variable signals for *ccrB1*, *ccrA3*, and *ccrB3* in the primer-directed protocol, while the random protocol yielded signals for *ccrB1* and *ccrB3* (Table 2). This suggested the possible presence of a novel *ccr* allotype in the MRSA isolates. No other known SCC or SCC*mec*-associated genes were detected in MRSA isolates M10/0061 and M10/0148 (Table 2).

DNA and amino acid sequence analysis of the genes encoding PBPs 1 to 4. Comparative analysis of the DNA sequences of the genes encoding PBPs 1 to 4 from CC130 MRSA isolates M10/0061 and M10/0148 and the CC130 MSSA isolate revealed that each gene in the MRSA isolates exhibited 100% DNA sequence identity to each other and to the corresponding genes in the MSSA isolate. The PBP sequences identified in M10/0061 by whole-genome sequencing were submitted to GenBank.

Identification of an SCC*mec* element encoding a novel *mecA* allotype in MRSA isolate M10/0061. Whole-genome sequencing of the ST130 MRSA isolate M10/0061 yielded 401 contigs ranging in size from ca. 100 bp to 100 kb, and 46 of these were >20 kb in size. The *ccr* genes were localized to a 90-kb contig that was found to contain sequences encoding a 30-kb SCC*mec* element which was analyzed in depth (Fig. 1). Because the present study focused on the 30-kb SCC*mec* element and immediately adjacent ORFs, other ORFs encoded by the 90-kb contig or other contigs are not discussed further here. Details of the ORFs identified within SCC*mec* of M10/0061 are described in Table S1 in the supplemental material. The SCC*mec* element harbored a novel allotype of the *mecA* gene designated $mecA_{M10/0061}$, the predicted protein from which exhibited 62% amino acid (aa) identity with MecA proteins previously described in MRSA and 63% aa identity with MecA from *Staphylococcus kloosii* and *Staphylococcus vitulinus*. Novel allotypes of the *mec* regulatory genes *mecI* and *mecR1* were also identified upstream of $mecA_{M10/0061}$, and they encoded proteins that exhibited 66% and 45% aa identities with proteins encoded by previously described MRSA *mecI* and *mecR1* genes, respectively (Fig. 1). A novel allotype of the β-lactamase resistance gene *blaZ* was identified downstream of *mecA* but still within the SCC*mec* element and exhibited 67% aa identity with the previously known *S. aureus blaZ* gene. The *ccrAB* genes within the SCC*mec* element from M10/0061 consisted of *ccrA1* and *ccrB3* genes and exhibited 86% and 93% aa identities to previously described *ccrA1* and *ccrB3* genes in the GenBank database, respectively.

The SCC*mec* element in M10/0061 was integrated at the same nucleotide position within *orfX* in M10/0061 as all other SCC*mec* elements described in MRSA isolates to date and was flanked by DR sequences (DR-1 and DR-2) that are characteristic of SCC*mec* elements (Fig. 1). A third DR (DR-3; Fig. 1) was identified outside the SCC*mec* region ca. 3 kb downstream of DR-2 in the novel SCC*mec* element. Three ORFs were identified within this 3-kb, region but they exhibited only weak similarity (39 to 53% aa identity) with ORFs in nonstaphylococcal species (Fig. 1; see Table S1 in the supplemental material).

No ORFs were identified in the 316-bp DNA sequence identified between *orfX* and *blaZ* in the SCC*mec* element from M10/0061; i.e., no ORFs were identified in the J3 region. The

J2 region (between the *mec* gene complex and the *ccr* genes) of the SCC*mec* element was ca. 4.5 kb and contained seven ORFs. For 6/7 ORFs, the highest similarities (49 to 91%) were to hypothetical proteins of *S. aureus* (2 of which were SCC*mec* related), *M. caseolyticus*, and *Staphylococcus saprophyticus*. For one ORF in the J2 region, the highest similarity was to a transposase of *Clostridium* spp., but these ORFs exhibited just 21% aa identity. The J1 region (between the *ccr* genes and DR-2) of the SCC*mec* element was ca. 16.5 kb, and 14 ORFs were identified. The highest similarities for any ORFs within the J1 region were of three ORFs (79 to 93% aa identity) to the arsenic resistance operon genes *arsC*, *arsB*, and *arsR*. For 2/14 ORFs (ORF_115 and ORF_16) in the J1 region, the highest similarity was to ORFs encoding hypothetical proteins described in SCC*mec* elements, one in MRSA and one in *Staphylococcus hominis*. Of the nine remaining ORFs within the J1 region, eight exhibited amino acid identities ranging from 39 to 89% to ORFs from CoNS, including a cassette chromosome helicase protein, a lipase precursor protein, a FAD-binding dehydrogenase, two ABC transporter proteins, a flavin reductase protein, a major facilitator superfamily protein, and a hypothetical protein. The remaining ORF (ORF_117) exhibited weak similarity (32% aa identity) with a DNA helicase in a nonstaphylococcal species.

Designation of the SCC*mec* element of M10/0061 as SCC*mec* XI. The website of the IWG-SCC (http://www.sccmec.org/Pages/SCC_TypesEN.html) lists a SCC*mec* type, designated SCC*mec* XI, from MRSA isolate LGA251 with a genetic organization within the *mec* and *ccr* complex genes similar to that of the SCC*mec* element identified in the present study in M10/0061. A search of the published literature and GenBank failed to yield descriptions of SCC*mec* XI. MRSA isolate LGA251 was originally recovered from a bovine source and has previously been assigned to ST425 (34) (http://www.sanger.as.uk/pathogens). The whole-genome sequence of LGA251 has been determined by the Wellcome Trust Sanger Institute (Cambridge, United Kingdom) and is available on the Sanger Institute website (http://www.sanger.as.uk/pathogens). Because the whole-genome sequence of LGA251 is not currently available in GenBank and has not yet been published, permission was obtained from the Sanger Institute to compare the SCC*mec* element identified in the human clinical MRSA isolate M10/0061 in the present study with that of SCC*mec* XI first identified by the Sanger Institute in bovine MRSA isolate LGA251. This comparative analysis revealed that both SCC*mec* elements exhibited 99.9% DNA sequence identity. Thus, we have designated the SCC*mec* type of M10/0061 XI (http://www.sccmec.org/Pages/SCC_TypesEN.html). Similarly, we have designated the *mec* complex of M10/0061, consisting of divergent *blaZ*, *mecA*, *mecR1*, and *mecI* genes, class E *mec*, reflecting the corresponding IWG-SCC designation for LGA251 (Fig. 1). The *ccrA1B3* gene combination of M10/0061 was designated type 8 *ccr* complex, again reflecting the corresponding IWG-SCC designation for LGA251 (Fig. 1).

No sequence similarity to the 3-kb region downstream of DR-2 in SCC*mec* $XI_{M10/0061}$ (Fig. 1) was identified downstream of SCC*mec* XI in LGA251.

Detection of SCC*mec* XI in M10/0148. PCR and sequencing revealed the presence of $ccrA1B3_{M10/0061}$ and $mecA_{M10/0061}$ as well as $mecI_{M10/0061}$, $mecR1_{M10/0061}$, and $blaZ_{M10/0061}$ in the second clinical CC130 MRSA isolate, M10/0148. Each of these genes exhibited 99 to 100% DNA sequence identity with the corresponding genes identified in M10/0061. The presence of the entire SCC*mec* XI element and the 3-kb region between DR-2 and DR-3 was also confirmed in M10/0148 using PCR and 10 primer pairs. M10/0148 yielded amplicons of the expected size using all primer pairs.

DISCUSSION

Methicillin-resistant *S. aureus* continues to be a significant problem in many hospitals worldwide, with potentially serious consequences for patients and significant extra demands on health care resources. Correct identification of MRSA is essential to ensure appropriate isolation and treatment of patients. Here we report on two MRSA isolates recovered from patients in Irish hospitals that exhibited phenotypic resistance to methicillin but were identified as MSSA using *mecA* PCR, a DNA microarray, the GeneXpert rapid real-time PCR assay, and a PBP latex agglutination assay. These two CC130 MRSA isolates harbored a 30-kb SCC*mec* element, designated SCC*mec* XI, that had several striking features, including highly divergent *mecA*, *mecI*, *mecR1*, *blaZ*, and *ccr* genes, and that was almost identical to an unpublished SCC*mec* element, designated SCC*mec* XI on the IWG-SCC website (http://www.sccmec.org/Pages/SCC_TypesEN.html). Each ORF identified within SCC*mec* element XI exhibited 21 to 93% aa identity to previously described ORFs in GenBank (see Table S1 in the supplemental material), indicating that this SCC*mec* element is distantly related to previously described SCC*mec* elements and may possibly represent an ancestral form of SCC*mec* in MRSA.

In all SCC*mec* elements that have been described previously in the literature, the *mec* complex genes (*mecA*, *mecI*, and *mecR1*) are almost identical. In contrast, the *mecA*, *mecR1*, and *mecI* genes identified in the CC130 MRSA isolates in the present study were highly divergent, exhibiting only 62%, 45%, and 66% aa identities, respectively, with the corresponding genes in GenBank. In addition, while *mecA* homologs have been described previously in *Staphylococcus kloosii*, *Staphylococcus sciuri*, *Staphylococcus vitulinus*, and *M. caseolyticus* (55, 65, 66, 73), the *mecA* gene in the CC130 isolates was also clearly distinct from each of these genes, exhibiting only 21 to 63% aa identity. The combination of *ccr* genes identified within SCC*mec* XI was also different from those previously described and was assigned a novel *ccr* type combination designated type 8.

Another unusual feature of the *mec* complex identified in SCC*mec* XI was the presence of *blaZ* upstream of *mecA*. The *blaZ* gene encodes a β-lactamase and is generally found as part of the *bla* operon (*blaI*, *blaR1*, and *blaZ*), which is widespread among Gram-positive bacteria. Apart from the *mec* complex identified in SCC*mec* XI in CC130 MRSA isolates in the present study and in bovine isolate LGA251 on the IWG-SCC website, the only previous report of a *mec* complex with *blaZ* was from *M. caseolyticus*, where a transposon harboring a *mec* gene complex with the same genetic organization as that in SCC*mec* XI (*blaZ*, *mecA*, *mecR1*, and *mecI*) was integrated downstream of *orfX* and was flanked by DRs (65). A non-*mecA* SCC element was integrated next to the *mec* transposon in *M.*

caseolyticus and was separated from *mecA* by DRs. It has been speculated previously that the *mec* gene complex was generated following integration of *mecA* into the *bla* operon and that the *blaZ-mecA-mecR1-mecI* complex in *M. caseolyticus* may represent the ancestral form of the *mec* gene complex in staphylococci (4, 63, 65). The *mec* complex of SCC*mec* XI may represent a putative ancestral *mec* gene complex in MRSA.

The J regions of SCC*mec* XI also differed significantly from those of previously described SCC*mec* elements. The J3 region of SCC*mec* XI was just 316 bp in size, which is substantially smaller than the J3 regions identified in SCC*mec* I to VIII. The J2 and J1 regions were more comparable in size to those of previously described SCC*mec* elements, but the 21 ORFs identified (excluding the *ccr* and *mec* complex genes) exhibited similarities to previously described ORFs ranging from only 21% to 93%. In addition, for only 4/21 ORFs the highest similarities (62 to 88%) were to genes previously identified in SCC*mec* elements in MRSA or CoNS. The remaining 17 ORFs exhibited similarities to non-SCC*mec* DNA sequences in *S. aureus* and CoNS and in some cases weak similarity to the sequences in nonstaphylococcal species. Of particular interest was the identification of genes with similarity (79 to 93% aa identity) to those of an arsenic resistance operon, consisting of *arsR*, a transcriptional repressor; *arsB*, an arsenite efflux pump; and *arsC*, an arsenate reductase. Both isolates exhibited resistance to sodium arsenite and sodium arsenate. Arsenic occurs naturally in the environment and is commonly found in the earth's crust; but it can also originate from agriculture and industry as a result of the use of pesticides, waste processing, and mining, and arsenic in drinking water is a problem in many parts of the world (47). Arsenic resistance genes have been identified in chromosomal and plasmid locations in many bacterial species, including *S. aureus*, CoNS, *Escherichia coli*, and *Pseudomonas fluorescens* (47). While genes encoding resistance to other heavy metals such as cadmium and mercury have been reported previously in SCC elements, including SCC*mer* in MRSA (22) and the SCC composite island in *S. epidermidis* strain ATCC 12228 (40), previously reported arsenic resistance genes in staphylococci have been plasmid located (47) and have not been associated with SCC*mec*. The very significant differences between SCC*mec* XI and previously described SCC*mec* elements suggest that SCC*mec* XI may have originated in another species or genus and are further evidence to support the theory that interspecies transfer of SCC*mec* may play a role in the emergence of novel MRSA strains.

It has been suggested previously that smaller SCC*mec* elements are more competitive than larger mobile genetic elements and that they may be more readily transferred between staphylococci (21, 31). The relatively small size of SCC*mec* XI (30 kb) suggests that it has the potential to become widespread among *S. aureus* and other staphylococci. In fact, MRSA strains harboring this novel SCC*mec* element may be more widespread than is currently known. First, this study identified SCC*mec* XI in CC130 MRSA clinical isolates from humans, and the Sanger Institute has also identified its presence in the ST425 bovine MRSA isolate LGA251 (http://www.sanger.as.uk/pathogens). This suggests that SCC*mec* XI has been horizontally transferred between two distinct *S. aureus* clones. However, the 3-kb region that was identified downstream of SCC*mec* in the CC130 isolates and that was flanked by DRs characteristic of SCC elements is absent in LGA251. This suggests that in the CC130 isolates SCC*mec* XI may have integrated adjacent to an existing SCC/SCC*mec* element that was subsequently partially lost, leaving behind this 3-kb SCC remnant. The weak similarity between the ORFs in this 3-kb region to unrelated nonstaphylococcal species suggests that another taxon may be the source of this possible SCC remnant. In addition, the CC130 MRSA isolates in the present study were not detected as MRSA using different *mecA* PCRs, the DNA microarray genotyping approach, or the GeneXpert MRSA assay, and therefore, there may be more MRSA strains harboring this SCC*mec* element that have not yet been detected. While confirmation of this hypothesis requires detailed molecular characterization of strains from human and animal sources, inclusion of the DNA sequences of these highly variant *mec* complex and *ccr* genes into the design of *mecA* PCR, DNA microarray, and rapid real-time PCR as well as antibody-based assays will aid the future detection of MRSA strains harboring this novel SCC*mec* element.

To the best of our knowledge, all CC130 isolates that have been reported to date have been MSSA and have mainly been reported from animals, including cows and rats (25, 64, 67). While in the present study there was no evidence of any association between the patients from whom the CC130 MRSA isolates were recovered and any animals, it is interesting to speculate that the patients may have acquired these MRSA strains from animal sources. First, it is very difficult to completely rule out a link between patient and animal sources, particularly livestock, pets, and raw meat products. The fact that Ireland has a large agriculture industry, particularly dairy and beef farming, coupled with the fact that the lineages (CC130 and ST425) in which SCC*mec* XI was identified have previously been found to be mainly associated with cattle, indicates that the CC130 MRSA isolates in the present study may have been acquired from bovine sources. Interestingly, the ST425 isolate LGA251 reported on the Sanger Institute website was recovered from a bovine source (http://www.sanger.as.uk/pathogens). In addition, the DNA microarray revealed the absence of β-toxin-converting bacteriophages in the two CC130 MRSA isolates. Previous studies have shown that β-toxin-converting bacteriophages are rare among *S. aureus* isolates from bovine sources (36) but common among those from humans (30, 38, 39). The zoonotic spread of MRSA has been reported previously, and this may contribute to the burden of MRSA strains among humans (68, 69).

It has been reported previously that mutations in several *S. aureus* genes, including *pbp4*, can result in high-level β-lactam resistance (5). However, comparative DNA sequence analysis of the PBP genes from the two CC130 MRSA isolates and the CC130 MSSA isolate revealed that there were no differences in the PBP gene sequences in the MRSA isolates and the MSSA isolate (which also lacked *mecA* and the SCC*mec* cassette), indicating that it is the novel *mecA* gene and not alterations in PBPs that is responsible for the methicillin resistance phenotype in these isolates. Further studies involving controlled expression of the novel *mecA* gene in methicillin-susceptible *S. aureus* laboratory strains are under way in order to provide further evidence that the novel *mecA* gene identified in CC130 MRSA is responsible for the methicillin resistance phenotype in these isolates.

The identification of a *mecA* gene in MRSA that is not detected by *mecA* PCR, a DNA microarray, the GeneXpert rapid real-time PCR assay, or a PBP latex agglutination assay is alarming. Thus, with regard to infection prevention and therapy, isolates harboring this SCC*mec* element should be treated as MRSA, even though they are identified by PCR to be *mecA* negative. Therefore, isolates in which unexplained resistance phenotypes are detected, such as methicillin resistance in apparently *mecA*-negative *S. aureus* isolates, require further characterization and should be submitted to specialized laboratories until updated routine assays are available. Routine procedures for MRSA screening need to be reevaluated, and phenotypic approaches such as the use of chromogenic media should not be abandoned.

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ADDENDUM IN PROOF

Following submission of our study for peer-review evaluation, we became aware that García-Álvarez et al. had submitted a paper for publication describing the identification and characterization of SCC*mec* type XI in the bovine ST425 MRSA isolate LGA251 (L. García-Álvarez et al., Lancet Infect. Dis., 2011. [Epub ahead of print.] doi:10.1016/S1473-3099(11)70126-8). This study was the first to identify the SCC*mec* XI element, the nomenclature for which was approved by the IWG-SCC.

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