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Origin and Molecular Evolution of the Determinant of Methicillin Resistance in Staphylococci▽

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important multidrug-resistant pathogens around the world. MRSA is generated when methicillin-susceptible *S. aureus* (MSSA) exogenously acquires a methicillin resistance gene, *mecA*, carried by a mobile genetic element, staphylococcal cassette chromosome *mec* (SCC*mec*), which is speculated to be transmissible across staphylococcal species. However, the origin/reservoir of the *mecA* gene has remained unclear. Finding the origin/reservoir of the *mecA* gene is important for understanding the evolution of MRSA. Moreover, it may contribute to more effective control measures for MRSA. Here we report on one of the animal-related *Staphylococcus* species, *S. fleurettii*, as the highly probable origin of the *mecA* gene. The *mecA* gene of *S. fleurettii* was found on the chromosome linked with the essential genes for the growth of staphylococci and was not associated with SCC*mec*. The *mecA* locus of the *S. fleurettii* chromosome has a sequence practically identical to that of the *mecA*-containing region (~12 kbp long) of SCC*mec*. Furthermore, by analyzing the corresponding gene loci (over 20 kbp in size) of *S. sciuri* and *S. vitulinus*, which evolved from a common ancestor with that of *S. fleurettii*, the speciation-related *mecA* gene homologues were identified, indicating that *mecA* of *S. fleurettii* descended from its ancestor and was not recently acquired. It is speculated that SCC*mec* came into form by adopting the *S. fleurettii mecA* gene and its surrounding chromosomal region. Our finding suggests that SCC*mec* was generated in *Staphylococcus* cells living in animals by acquiring the intrinsic *mecA* region of *S. fleurettii*, which is a commensal bacterium of animals.

The first methicillin-resistant *Staphylococcus aureus* (MRSA) isolate was reported in England in 1961, soon after the introduction of methicillin (10). Since then, it has become an important pathogen in both health care settings and communities around the world (27). MRSA is generated when methicillin-susceptible *S. aureus* (MSSA) exogenously acquires a staphylococcal cassette chromosome *mec* (SCC*mec*) (9). SCC*mec* has been identified not only in *S. aureus* but also in other coagulase-positive and coagulase-negative staphylococci (8). SCC*mec* is speculated to be transmissible among staphylococcal species as a mobile element; however, its origin/reservoir has not been clarified yet (7). Finding the origin/reservoir of the *mecA* gene is important for understanding the evolution of MRSA. Moreover, it may contribute to more effective control measures for MRSA. There are various types and subtypes in SCC*mec*, but they are made up of the following two essential components: (i) the *mec* gene complex, containing the *mecA* gene encoding the penicillin-binding protein 2′ (PBP2′) with reduced affinity to beta-lactam antibiotics, and (ii) the *ccr* gene complex, encoding site-specific recombinase(s) for the movement of the element. By the action of *ccr* gene-encoded recombinases, SCC*mec* is excised from the chromosome and site-specifically integrated at the 3′ end of *orfX* (11), an open reading frame (ORF) of unknown function, which is located near the replication origin (*oriC*) on the chromosome (13). A large number of SCC*mec* elements have been sequenced and classified according to the combination of types of *mec* and *ccr* gene complexes (8). Although nucleotide diversity of *ccr* genes identified in various *Staphylococcus* species has been reported, *mecA* genes contained in SCC*mec* are almost identical regardless of the *Staphylococcus* species carrying them (8). *mec* gene complexes are classified into 4 classes (classes A, B, C, and D) by the presence or absence of certain insertion sequences (ISs) in the *mecR1* gene, but all *mec* gene complexes contained IS*431mec* (also called IS*431R*) downstream of the *mecA* gene (Fig. 1) (8, 12). The class A *mec* gene complex is regarded as the prototypic structure, having the gene order IS*431mec-mecA-mecR1-mecI.* Class B, C, and D *mec* gene complexes are considered to be derived from the class A *mec* gene complex (8, 12). The region between the *mecA* gene and IS*431mec* is called a hypervariable region (HVR) (19), which can be used for genotyping of MRSA strains (21). The HVR contains a truncated *mvaS* gene encoding the 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) synthase, a varied number of direct repeat units (DRUs) that is responsible for the length polymorphism of the *mec* gene complex (19), an intact *ugpQ* gene encoding glycerophosphoryl diester phosphodiesterase, and an intact *maoC* gene encoding the acyl dehydratase MaoC (Fig. 1). Some strains, however, lack some of the above-mentioned constituents of the HVR (16, 24).

In staphylococci, previously, two *mecA* gene homologues with 80% and 91% nucleotide identities have been found in *Staphylococcus sciuri* and *Staphylococcus vitulinus* species, respectively (5, 22). The *mecA* gene homologue of *S. sciuri* has been considered to have a ubiquitous presence among its species and to be the evolutionary precursor of the *mecA* gene (5, 32). However, the *mecA* gene homologues found in their chro-

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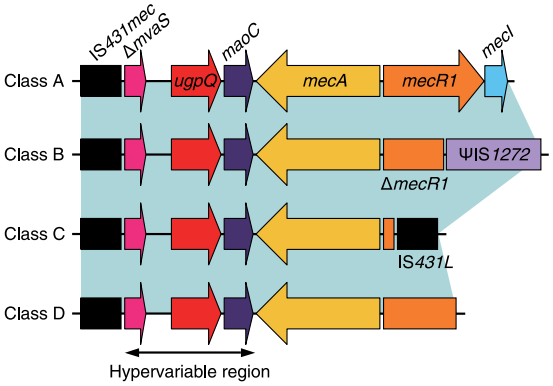

FIG. 1. Basic structure of the representative *mec* gene complex. The class A *mec* gene complex is composed of intact *mecR1* and *mecI*, encoding the signal transducer and repressor for the *mecA* gene, respectively, upstream of the *mecA* gene without integration of an IS element. The class B and C *mec* gene complexes have ψIS*1272* and IS*431L* integrated in *mecR1*, respectively, which results in the partial deletion of *mecR1* and complete deletion of the *mecI* genes. Class D has no IS element, but a part of *mecR1* and complete *mecI* genes are deleted. All *mec* gene complexes contained IS*431mec* (also called IS*431R*) downstream of the *mecA* gene.

mosomes were not the constituents of the *mec* gene complex or SCC*mec*. We speculated that the origin/reservoir of the *mecA* gene would be found in a staphylococcal species closely associated with *S. sciuri* and *S. vitulinus*. These species belong to the *S. sciuri* species group among the genus *Staphylococcus.* The group is composed of the following four species, which are resistant to novobiocin and catalase production and coagulase nonproduction: *S. sciuri*, *S. vitulinus*, *Staphylococcus lentus*, and *Staphylococcus fleurettii* (20, 25, 29, 30). They are usually isolated from a variety of animals and food products of animal origin and not frequently isolated from humans (20, 23, 25, 29, 30).

We isolated staphylococcal strains of the *S. sciuri* species group from an animal source, tested for the presence of the *mecA* gene and its homologues, and determined the nucleotide sequences of the chromosome regions around the *mecA* gene and its homologues.

MATERIALS AND METHODS

Bacterial strains. *S. sciuri* species group strains used in this study are shown in Table 1. We identified bacterial strains by determination of the *hsp60* and/or *sodA* sequences, as described in previous reports (14, 17). We also used *Staphylococcus kloosii* strain ATCC 43959^T and *Staphylococcus xylosus* strain ATCC 29971^T to determine the *mvaS* gene sequences.

DNA manipulations. Preparation of DNA, inverse PCR, nested PCR, nucleotide sequence determination, and alignment were based on previously published protocols (9, 33). Detection of *mecA* genes and their homologues was carried out by degenerate PCR, using synthetic primers MECA1 and MECA2 with the nucleotide sequences 5′-CCWMAAACWGGHGAAYTVTTAGC-3′ and 5′-CCYTGKCCRTAWCCTGARTCWGC-3′, respectively. These primers were designed by multiple alignments of the nucleotide sequences of the *mecA* genes of SCC*mec*, the *mecA* gene homologues of *S. sciuri* and *S. vitulinus*, and the *mecA* gene of *Macrococcus caseolyticus* (*mecA*$_{Mc}$), obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/). The PCR mixture contained 100 ng DNA extract, 10 pmol of each primer, 1× PCR buffer, 0.2 mM of each deoxynucleoside triphosphate (dNTP), and 2 U Ex *Taq* (Takara Co., Ltd., Kyoto, Japan). The PCR thermal cycling conditions used were 1 cycle at 95°C for 2 min, followed by 30 cycles at 94°C for 30s, 50°C for 30s, and 72°C for 1 min, with a final cycle at 72°C for 2 min. The sequencing analyses of the PCR products were preformed.

The nucleotide sequences of the *mvaS* genes of *S. aureus*, *Staphylococcus epidermidis*, *Staphylococcus capitis*, *Staphylococcus warneri*, *Staphylococcus saprophyticus*, *Staphylococcus haemolyticus*, and *Staphylococcus hominis* were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/). A set of primers, MSC1 and MSC2, with nucleotide sequences 5′-GGTIATHGTDGCA ACWGARTCWG-3′ and 5′-CCCATTTTDGTRAAWGGWACRTGG-3′, respectively, were designed by multiple alignment of nucleotide sequences of these *mvaS* genes. By using the primers, we performed degenerate PCR to amplify the *mvaS* genes of *S. xylosus*, *S. kloosi*, and *S. lentus*. The PCR mixture contained 100 ng DNA extract, 10 pmol of each primer, 1× PCR buffer (Mg^{2+} free), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 2 U Ex *Taq* (Takara Co., Ltd., Kyoto, Japan). The PCR thermal cycling conditions used were 1 cycle at 95°C for 3 min, followed by 35 cycles at 94°C for 30s, 37°C for 30s, and 72°C for 1 min, with a final cycle at 72°C for 2 min. The sequencing analyses of the PCR products were preformed before the inverse PCR in order to determine the complete sequences of the *mvaS* genes.

Computer analysis. ORFs were extracted with genome analysis-oriented software In Silico Molecular Cloning version 1.5 (In Silico Biology, Yokohama, Japan). Homology searches were performed using the Internet-available BLAST program from the National Center for Biotechnology Information, United States of America (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis of *mvaS*, 16S rRNA, and *mecA* genes. Phylogenetic analysis of *mvaS* genes of staphylococci was performed, with the *mvaS* gene nucleotide sequence of *Listeria monocytogenes*, obtained from GenBank database, as an outgroup. The nucleotide sequences of 16S rRNA of *S. aureus*, *S. epidermidis*, *S. capitis*, *S. warneri*, *S. saprophyticus*, *S. haemolyticus*, *S. hominis*, *S. sciuri*, *S. vitulinus*, *S. lentus*, *S. fleurettii*, *S. kloosii*, *S. xylosus*, *Staphylococcus carnosus*, and *L. monocytogenes* (outgroup) were also obtained from the GenBank database. Phylogenetic analysis of *mecA* genes and its homologues was performed by using the *mecA*$_{Mc}$ gene as an outgroup. The phylogenetic tree was generated by the CLUSTALW program using the neighbor-joining method (http://clustalw.ddbj.nig.ac.jp/top-j.html). The tree was visualized with TreeView software, Glasgow, United Kingdom (http://taxonomy.zoology.gla.ac.uk/rod /treeview.html).

Detection of *xyl* genes and xylose fermentation testing for *S. sciuri*. Detection of the *xylA-xylB* gene of *S. sciuri* was carried out by PCR using primers XAS-F, 5′-GTGTTGCATACTGGCATAC-3′, and XBS-R, 5′-GCACCAATTTTAGAC TGGG-3′, which amplify an anticipated 2,190-bp fragment. PCR was based on previously published protocols (12). Xylose fermentation tests were performed using purple agar base (Difco, Detroit, MI) with or without D-xylose at a 1% concentration. A colony was inoculated in the stab with an inoculating needle and incubated at 37°C for 48 h.

Detection and sequencing analyses of the regions between the *mvaS* and *xylR* genes and the *xylR* and *xylE* genes of *S. vitulinus*. Detection of the region between the *mvaS* and *xylR* genes of *S. vitulinus* was performed by PCR using primers MVAV-1, 5′-CCAGAGAACACTTTGTATG-3′, and XYRV-1, 5′-GCATACC CCATAAGTCATTTTG-3′. Detection of the region between the *xylR* and *xylE* genes of *S. vitulinus* was performed by PCR using primers XYRV-2, 5′-GAAG CTATTAAGAACGAGTTC-3′, and XYEV-1, 5′-CCTACTCCAATACCACC G-3′. PCR and sequencing analyses were based on previously published protocols (12).

Antimicrobial susceptibility testing. MICs were determined by the agar dilution method using Mueller-Hinton agar (Difco, Detroit, MI), as recommended by the CLSI guidelines (3). The MIC breakpoints for resistance used were those recommended in the CLSI guidelines (4). The antibiotics tested were as follows: oxacillin, cefoxitin, and ceftriaxone (Sigma Chemical Co., Ltd., St. Louis, Mo); ampicillin (Pfizer, Inc.); and ceftizoxime (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan).

RESULTS AND DISCUSSION

Detection of the *mecA* genes and its homologues in the *S. sciuri* species group. All 3 strains of *S. sciuri*, 6 of 20 strains of *S. vitulinus*, and 8 of 10 strains of *S. fleurettii* were found to be *mecA* positive by PCR analysis using the degenerate primers for amplifying the *mecA* genes and its homologues (Table 1). Sequencing analysis of the PCR products (512 bp) revealed that the nucleotide identities of the *mecA* genes detected in *S. sciuri*, *S. vitulinus*, and *S. fleurettii* strains shared with the *mecA*

TABLE 1. Characterization of the *S. sciuri* species group strains used in this study

Species	Strain	Source	*mecA* homologue	MIC (μg/ml)					Type[a]	No. of DRUs[b]
				Oxacillin	Ampicillin	Ceftizoxime	Ceftriaxone	Cefoxitin		
S. fleurettii	SFMP01 (CCUG 43834^T)	Cheese (made of goat milk)	$mecA_{Sf}$	64	1	32	16	2	A	5
	SFMP02	Chicken meat	$mecA_{Sf}$	8	1	32	16	1	A	5
	SFMP03	Chicken meat	$mecA_{Sf}$	128	1	128	64	8	A	5
	SFMP04	Chicken meat	$mecA_{Sf}$	128	1	64	64	4	A	5
	SFMP05	Chicken meat	$mecA_{Sf}$	32	0.25	4	8	1	A	10
	SFMP06	Cock	$mecA_{Sf}$	64	1	32	32	2	B	0
	SFMP07	Cat	$mecA_{Sf}$	8	0.25	4	8	1	B	0
	SFMP08	Chicken meat	$mecA_{Sf}$	64	1	32	16	2	C	1
	SFMN01	Horse	ND[c]	0.5	0.125	2	4	2	D	0
	SFMN02	Horse	ND	0.5	0.125	2	4	2	E	0
S. sciuri subsp. *carnaticus*	ATCC 700058^T	Eastern gray squirrel	$mecA_{Ss}$	1	0.125	2	8	2		
S. sciuri subsp. *sciuri*	ATCC 29062^T	Veal leg, sliced	$mecA_{Ss}$	0.5	0.125	2	8	2		
S. sciuri subsp. *rodentium*	ATCC 700061^T	Norway rat	$mecA_{Ss}$	0.5	0.125	2	8	2		
S. vitulinus	SVMP01	Horse	$mecA_{Sv}$	0.5	<0.063	0.5	4	2		
	SVMP02	Horse	$mecA_{Sv}$	0.5	<0.063	0.5	2	1		
	SVMP03	Horse	$mecA_{Sv}$	0.5	<0.063	0.5	4	2		
	SVMP04	Horse	$mecA_{Sv}$	0.5	<0.063	0.5	2	1		
	SVMP05	Horse	$mecA_{Sv}$	0.5	<0.063	1	4	1		
	SVMP06	Horse	$mecA_{Sv}$	0.5	<0.063	0.5	2	1		
	SVMN01 (ATCC 51145^T)	Ground lamb	ND	0.5	0.125	4	8	2		
	SVMN02	Horse	ND	0.5	<0.063	1	4	1		
	SVMN03	Horse	ND	0.5	<0.063	0.25	1	0.5		
	SVMN04	Horse	ND	0.5	<0.063	0.5	2	1		
	SVMN05	Horse	ND	0.25	<0.063	0.5	1	1		
	SVMN06	Horse	ND	0.5	<0.063	0.5	4	2		
	SVMN07	Horse	ND	0.5	<0.063	0.5	2	2		
	SVMN08	Horse	ND	0.5	<0.063	0.25	0.25	0.25		
	SVMN09	Horse	ND	0.5	<0.063	0.5	2	1		
	SVMN10	Horse	ND	0.5	<0.063	0.25	1	0.5		
	SVMN11	Horse	ND	0.5	0.125	1	4	1		
	SVMN12	Horse	ND	0.5	<0.063	0.5	1	1		
	SVMN13	Horse	ND	0.5	<0.063	0.25	1	1		
	SVMN14	Horse	ND	0.5	<0.063	0.5	2	0.5		
S. lentus	ATCC 29070^T	Goat udder	ND	0.5	<0.063	1	2	2		

[a] Genomic structure types of *S. fleurettii*.
[b] DRUs separated by an IS element were counted as one DRU.
[c] ND, not detected.

gene of MRSA strain N315 were 85 to 86%, 94%, and 99 to 100%, respectively. The PCR did not detect the *mecA* genes or *mecA* gene homologues in 10 independent *S. lentus* strains (data not shown).

Genomic structure analysis of the *mecA* gene region of *S. fleurettii*. We performed genome analysis around the *mecA* gene by using inverse PCR-based sequencing analysis. Surprisingly, we found that *S. fleurettii* had an almost identical structure to that of the class A *mec* gene complex in SCC*mec* with an intact *mvaS*, which is not truncated by IS*431mec* found in MRSA strain N315 (Fig. 2a). Moreover, the intact *mvaS* gene was followed by the *mvaAC* genes, constituting the mevalonate pathway (Fig. 2a) (31). Between the *mvaS* and *mecA* genes on the chromosome of *S. fleurettii*, the *ugpQ* and *maoC* genes, similar to those downstream of the *mecA* gene in SCC*mec*, were present. Upstream of *mecA-R-I* in *S. fleurettii*, genes encoding a phenol-soluble modulin *mec* (*psm-mec*) (18), xylose repressor (*xylR*), metallo-beta-lactamase family protein, rhodanase domain-containing protein, and two hypothetical proteins were present. This is also consistent with the corresponding region of the SCC*mec* of MRSA strain N315. In regard to the genetic organization following it, however, there were differences between *S. fleurettii* and the N315 SCC*mec* (Fig. 2a). In the *S. fleurettii* chromosome, *xylABE* encoding xylose isomerase, xylulokinase, and the xylose transporter, respectively, were identified, rather than Tn*554* (encoding macrolide and streptomycin resistance) found in the N315 SCC*mec*, indicating that the *xylR* gene constituted an *xyl* operon with *xylABE* (26). Conversely, it became clear that the *xylR* gene present upstream of the class A *mec* gene complex was origi-

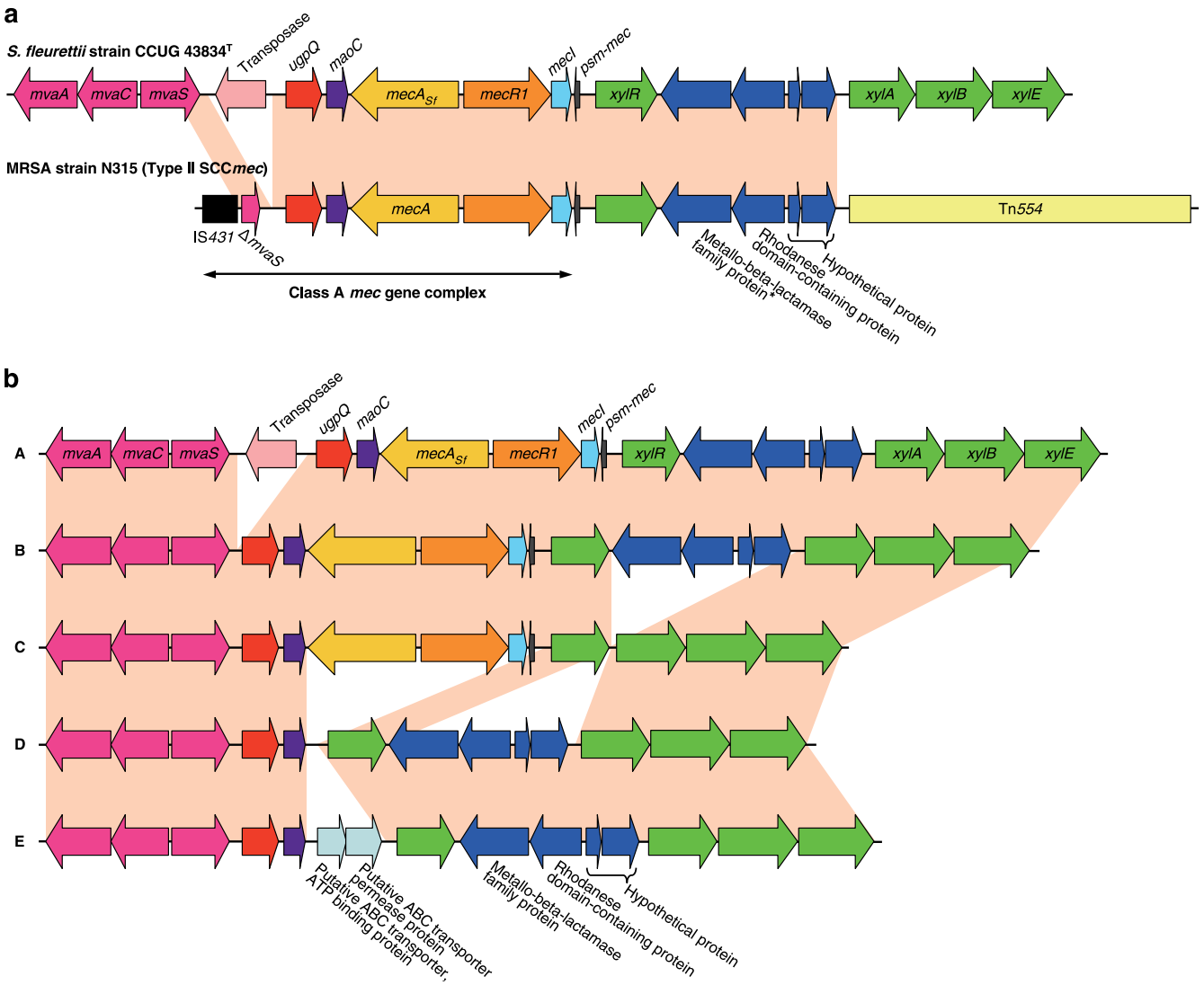

FIG. 2. Genomic organization of the $mecA_{Sf}$ gene locus of *S. fleurettii*. (a) Comparison to the locus-containing type II SCC*mec* of MRSA strain N315. The $mecA_{Sf}$-positive *S. fleurettii* possessed a stretch of chromosome sequence that was practically identical with that of MRSA SCC*mec*, spanning the class A *mec* gene complex and adjacent chromosomal region containing *psm-mec*, *xylR*, the genes encoding the metallo-beta-lactamase family protein, the rhodanase domain-containing protein, and other hypothetical proteins. The *mvaS* gene of *S. fleurettii* existed in intact form without insertion of IS*431*. Moreover, the *xyl* operon was identified on the *S. fleurettii* chromosome from which *xylR* of SCC*mec* must have originated. The entire corresponding regions of the two chromosomes are colored in pink. Arrows indicate the genes and their directions of transcription. Truncated *mvaS* is shown by Δ*mvaS*. *, The gene encoding the metallo-beta-lactamase family protein of MRSA strain N315 was a pseudogene with a nonsense mutation (ochre) incorporated next to codon 312. (b) Structural diversity observed in the $mecA_{Sf}$ gene loci of *S. fleurettii* strains. Five types (A, B, C, D, and E) were identified. The regions with practically identical nucleotide sequences across the *mva* and *xyl* genes are colored in pink. Arrows indicate the genes and their directions of transcription.

nally a part of the *xyl* operon. The *mecA* gene of *S. fleurettii* present at this gene locus is designated $mecA_{Sf}$. This locus (Fig. 2a, pink) shared 99% nucleotide identity with the corresponding locus of type II SCC*mec* carried by MRSA strain N315. This correlation is found not only in MRSA strain N315 but also in strains carrying SCC*mec* type II, III, and VIII containing the class A *mec* gene complex. Such strains have been disseminated around the world. In reference to the GenBank database, this correlation is found in, for example, SCC*mec* type II of MRSA strains N315 from Japan, MRSA252 from United Kingdom, and 04-02981 from Germany and methicillin-resistant *S. epidermidis* strain RP62A from the United States; SCC*mec* type III of MRSA strains TW20 from United Kingdom, 85/2082 from New Zealand, and BK16704 from Romania and methicillin-resistant *Staphylococcus pseudintermedius* strains KM241 and KM1381 from Switzerland; and SCC*mec* type VIII of MRSA strain C10682 from Canada. In SCC*mec* type III, ψTn*554* (encoding cadmium resistance) is present, rather than Tn*554* found in SCC*mec* type II and VIII (8).

Sequencing analysis of the chromosomes of *S. fleurettii* strains revealed five structural diversities (Fig. 2b). Type A (5/10 strains, including strain CCUG 43834^T) had an IS element between the *mvaS* and *ugpQ* genes (42% amino acid

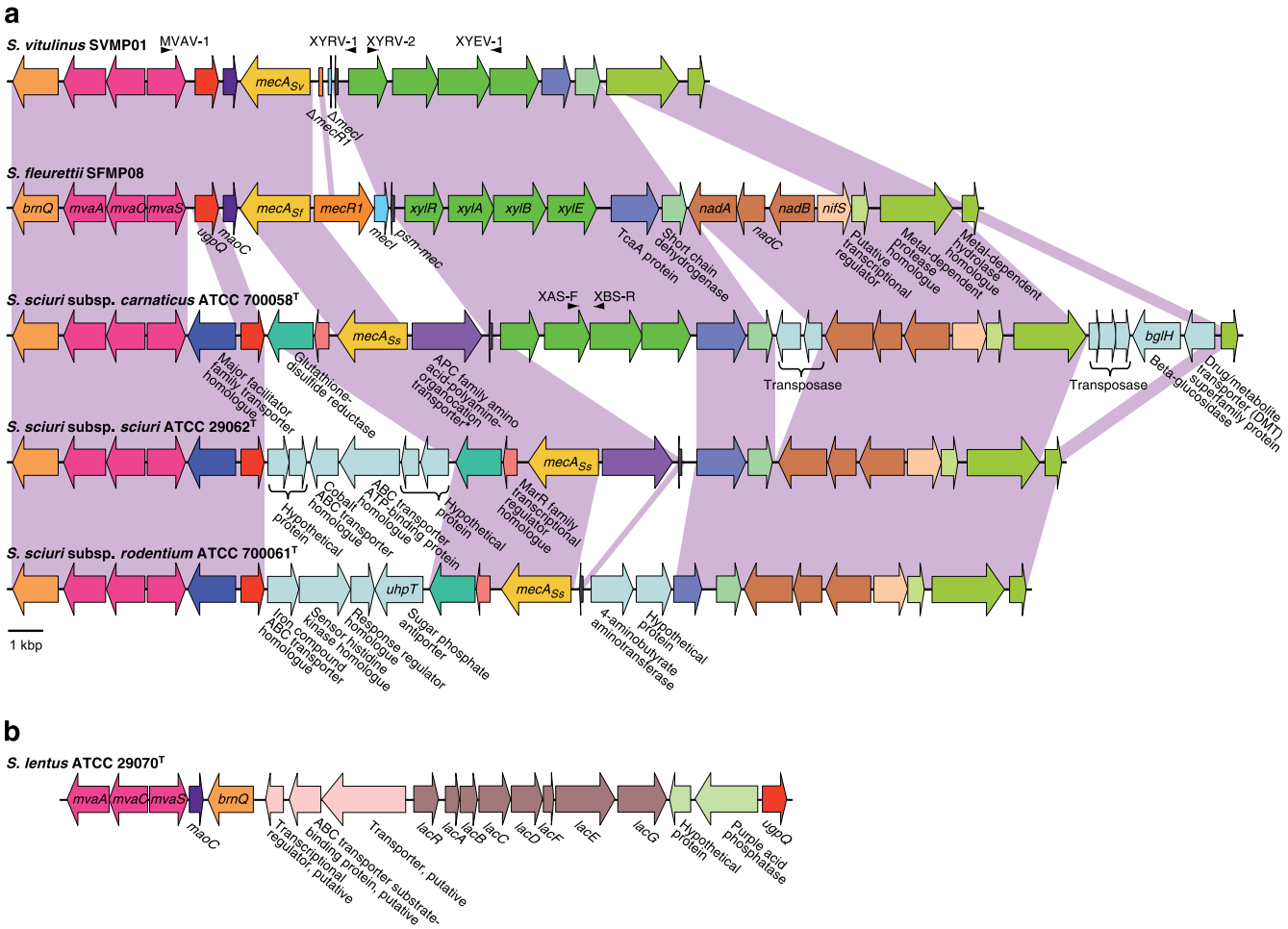

FIG. 3. Genomic structure analysis of the *S. sciuri* species group. (a) The *mecA* gene homologue-containing chromosomal regions of *S. vitulinus*, *S. sciuri* subsp. *carnaticus*, *S. sciuri* subsp. *sciuri*, and *S. sciuri* subsp. *rodentium* compared to those of *S. fleurettii.* The corresponding regions among the species (and subspecies) are colored in light purple. Arrows indicate the genes and their directions of transcription. Noncorresponding genes found in these regions are shown with pale blue arrows. Truncated *mecR1* and *mecI* are shown by Δ*mecR1* and Δ*mecI*, respectively. The positions of primers (MVAV-1, XYRV-1, XYRV-2, XYEV-1, XAS-F, and XBS-R) are shown with arrowheads. The scale bar is not necessarily correlated with the noncoding region. *, The gene encoding the APC family amino acid-polyamine-organocation transporter of *S. sciuri* subsp. *carnaticus* strain ATCC 700058^T was a pseudogene due to an early stop codon caused by a frameshift. (b) Genomic structure of the *mvaSCA* gene locus of *S. lentus*. Arrows indicate the genes and their directions of transcription.

identity and 62% amino acid similarity to IS*256*). The IS was integrated into one of the DRUs. Type B (2/10 strains) had no IS element between the *mvaS* and *ugpQ* genes. In type C (1/10 strains), the *xyl* operon was present in its original form, *xylRABE*, without the insertions between *xylR* and *xylA*. One of the two $mecA_{Sf}$-negative strains (type D) lacked *mecR1*, *mecI*, and *psm-mec*, and thus, the *maoC* and *xylR* genes were present side-by-side. The other $mecA_{Sf}$-negative strain (type E) possessed genes encoding a putative ABC transporter ATP-binding protein and an ABC transporter permease protein (57% and 62% amino acid similarity to SERP0984 and SERP0983 of *S. epidermidis* strain RP62A, respectively) between the *maoC* and *xylR* genes. In eight $mecA_{Sf}$-positive *S. fleurettii* strains, the regions between the *mvaS* and *mecA* genes, which corresponded to the HVR of the *mec* gene complex, showed the length polymorphism according to the varied number of DRUs (Table 1) and integration of the IS element.

Genomic structure of the *mecA* gene homologue regions of *S. sciuri* and *S. vitulinus* in comparison with that of *S. fleurettii*. We performed genome analysis around the *mecA* gene homologues of *S. sciuri* and *S. vitulinus* by using inverse PCR-based sequencing analysis. Compared with the genomic structure of *S. fleurettii*, similar genomic structure was found in the chromosomal region extending over 20 kbp in size around each *mecA* gene homologue (Fig. 3a). The *mecA* gene homologues of *S. sciuri* and *S. vitulinus* present at the corresponding loci are designated $mecA_{Ss}$ and $mecA_{Sv}$, respectively. Downstream of $mecA_{Sf}$, $mecA_{Sv}$ and $mecA_{Ss}$, the *ugpQ*, *mvaACS*, and *brnQ* genes encoding a branched chain amino acid ABC transporter were identified in all the three species. A $mecA_{Sv}$-positive *S. vitulinus* strain possessed truncated remnants of the *mecR1* and *mecI* genes upstream of $mecA_{Sv}$. We confirmed that all 6 $mecA_{Sv}$-positive *S. vitulinus* strains possessed practically identical $mecA_{Sv}$ genes and truncated *mecR1* and *mecI* genes by

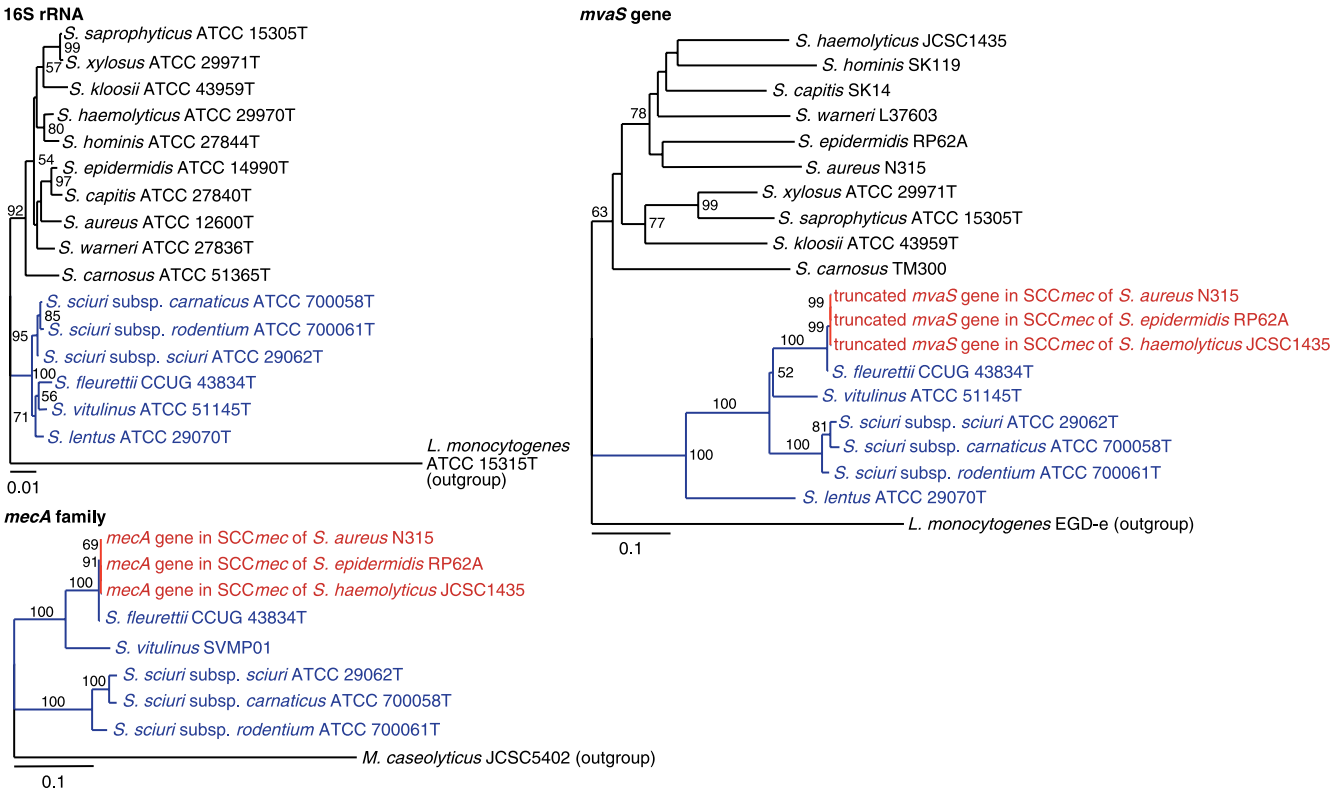

FIG. 4. Phylogenetic analysis of 16S rRNA, *mvaS*, and *mecA* genes. Strain names are preceded by the names of the bacterial species. The branch length indicates the distance, which is expressed as the number of substitutions per 100 bases. Numbers at the branching points represent the percent occurrence in 1,000 random bootstrap replications of neighbor-joining analyses. Values of less than 50% are not shown. The sizes of *mvaS* genes varied from 1,167 to 1,179 bp, except for the truncated *mvaS* gene (369 bp) present in SCC*mec*. The genes identified in SCC*mec* and obtained from the *S. sciuri* species group are colored red and blue, respectively.

conducting PCR testing followed by nucleotide sequencing, whereas $mecA_{Sv}$-negative *S. vitulinus* strains were negative for these genes. Thus, in their chromosomes, *maoC* and *psm-mec* genes were found adjacent to each other. The adjacent *xyl* operon was well conserved in all 20 strains of *S. vitulinus*.

S. sciuri did not carry *mecR1* and *mecI* genes upstream of $mecA_{Ss}$ in any of its three subspecies. There were considerable differences in the genomic organization around $mecA_{Ss}$ genes among three subspecies (Fig. 3a). We identified the *xyl* operon in *S. sciuri* subsp. *carnaticus* strain ATCC 700058^T but not in *S. sciuri* subsp. *sciuri* strain ATCC 29062^T or *S. sciuri* subsp. *rodentium* ATCC 700061^T on the corresponding locus. We confirmed the absence of the *xyl* operon in the latter two strains by PCR and xylose fermentation testing. Besides the *xyl* operon, differentiations of the three *S. sciuri* subspecies were accompanied by the insertion of some transporter genes. *S. sciuri* strains have been isolated from various sources, including animals, humans, and the environment (5). The ability of *S. sciuri* to survive in a wide range of environments may correlate with its intraspecies diversity such as the genomic structures observed in the $mecA_{Ss}$ chromosome locus.

In all the three tested species, genes encoding lipoprotein (TcaA protein), short-chain dehydrogenase, metal-dependent protease, and metal-dependent hydrolase were commonly identified downstream of $mecA_{Sf}$, $mecA_{Sv}$, and $mecA_{Ss}$ in identical gene order and orientation. Between genes encoding short-chain dehydrogenase and metal-dependent protease, *nadA* (encoding quinolinate synthetase), *nadB* (encoding L-aspartate oxidase), *nadC* (encoding nicotinate-nucleotide pyrophosphorylase), and *nifS* (encoding quinolinate synthetase) and a gene encoding a putative transcriptional regulator were commonly identified in *S. fleurettii* and *S. sciuri* but not in *S. vitulinus*. The genomic structure analysis of the chromosomal locus indicated that the *mecA* genes in this locus of the *S. sciuri* species group are descendants of the ancestral *mecA* gene carried by the common ancestor of *S. fleurettii*, *S. vitulinus*, and *S. sciuri.* The molecular differentiation of the archaic *mecA* gene into three alleles, $mecA_{Sf}$, $mecA_{Sv}$, and $mecA_{Ss}$, must have occurred by evolutionary processes during speciation of the ancestor bacterium into each staphylococcal species.

Phylogenetic analysis. The *mvaACS* genes encode enzymes of the mevalonate pathway, which are involved in the biosynthesis of isopentenyl diphosphate (IPP) (31). In staphylococci, the mevalonate pathway is composed of six genes, *mvaC*, *mvaS*, *mvaA*, *mvaK1*, *mvaK2*, and *mvaD*. These genes encode acetyl-CoA acetyltransferase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, and mevalonate diphosphate decarboxylase, respectively. All genes are essential for the growth of staphylococci (31). We performed the phylogenetic analysis of the *mvaS* genes of staphylococci. The topology of *mvaS* gene-derived tree was similar to that obtained by analyzing 16S rRNA genes (Fig. 4).

The *mvaS* genes of the *S. sciuri* species group formed a separate cluster from the other staphylococci, which was analogous to that formed in the 16S rRNA-based tree. This confirmed the vertical transmission of *mvaS* gene from the ancestral bacterium. The tree also showed that *mvaS* of *S. fleurettii* was most closely related to the truncated *mvaS* gene present within the *mec* gene complex in SCC*mec* (Fig. 4). The nucleotide identity between them was 99.7%. Furthermore, the topology of the *mecA* gene and its homologue-derived tree was correlated with that of the *S. sciuri* species group, obtained by analyzing *mvaS* genes (Fig. 4). These results suggested that the archaic *mvaS* gene evolved with the archaic *mecA* gene, side-by-side with the chromosome locus of the ancestral bacterium. Therefore, our study indicated that *S. fleurettii* had inherited the *mecA* gene from its ancestor by the vertical transmission and had not recently acquired it.

The *mvaS* gene of *S. lentus*, the fourth member of the *S. sciuri* species group, was somewhat distant from those of the other *S. sciuri* species group (Fig. 4). Genomic analysis of the *mva* gene locus of *S. lentus* also showed a genomic organization different from those of the other *S. sciuri* species group (Fig. 3b). By analyzing 10 independent strains, we identified the identical genomic structures upstream of the *mva* gene locus (data not shown). PCR did not detect the *mecA* gene homologue using whole cellular DNAs as templates. Therefore, the archaic *mecA* gene might have been carried by the common ancestor of *S. sciuri*, *S. fleurettii*, and *S. vitulinus* but not of *S. lentus*. Alternatively, *S. lentus* might have lost its *mecA* gene homologue at a certain stage of its evolutionary history.

Comparison of antibiotic resistance. We determined beta-lactam MICs for the *S. sciuri* species group strains. $mecA_{Sf}$-positive *S. fleurettii* strains had clearly higher MIC values for oxacillin, ceftizoxime, and ceftriaxone than $mecA_{Sf}$-negative *S. fleurettii* strains. On the other hand, none of the three $mecA_{Ss}$-carrying *S. sciuri* strains or $mecA_{Sv}$-positive *S. vitulinus* strains exhibited resistance to beta-lactam antibiotics (Table 1). Expression of the *mecA* gene homologue of *S. sciuri* ($mecA_{Ss}$) has been intensively studied (1). Although *S. sciuri* strains are uniformly susceptible to beta-lactam antibiotics, only its up-regulated form *in vitro* or clinical isolates with a mutation or an IS element in the promoter region of $mecA_{Ss}$ showed beta-lactam resistance. In contrast, $mecA_{Sf}$ confers methicillin resistance, as it has a nucleotide sequence practically identical to that of the *mecA* gene of MRSA strain N315 (99.8% nucleotide identity compared to that of the $mecA_{Sf}$ gene of *S. fleurettii* strain CCUG 43834^T). We speculated that the species *S. fleurettii* developed the $mecA_{Sf}$ gene in an environment where beta-lactam antibiotics frequently served as selective pressure during the speciation process.

Many studies on the function of the *mecA* gene have been done since the first discovery of MRSA in 1961. However, the origin/reservoir of the *mecA* gene has remained a mystery for half a century. Finally, we found in *S. fleurettii* the original chromosomal locus that must have served as the template for the *mec* gene complex of SCC*mec*. It is a commensal bacterium of animals. The formation of SCC*mec* seems to have occurred by a combination of the following two genetic components: the *S. fleurettii*-derived *mec* gene complex and an SCC element without the *mecA* gene. For the excision of the *mec* gene complex from the *S. fleurettii* chromosome, IS*431mec* might have been involved, because IS*431* has been found as part of many composite transposons (2). It is controversial as to whether the new SCC*mec* is being frequently produced around the world even now or if the generation of SCC*mec* occurred in only a few events in the past before a limited number of SCC*mec* spread around the world and were modified into the extant genetic diversity of SCC*mec*. Recently, we reported a suggestive case for the formation of an SCC*mec*-like element from the two genetic components occurring in *Macrococcus caseolyticus* strains isolated from chicken meat (28). Macrococci are closely related to staphylococci and are also commensal bacteria of animals. The resultant SCC*mec*-like element was different from SCC*mec* of MRSA in terms of nucleotide sequence and genomic organization of the *mec* gene complex. However, this observation raises the possibility that the generation of the new SCC*mec* is also ongoing in staphylococci. In either case, *S. fleurettii* is considered to be the origin of *mec* gene complex. However, *S. fleurettii* itself may not form SCC*mec* on its chromosome, because it is intrinsically resistant to beta-lactam antibiotics due to the carriage of the $mecA_{Sf}$ gene on the chromosome. Hence, other methicillin-susceptible animal-borne *Staphylococcus* species coexistent with *S. fleurettii* would be more likely candidates for the formation of SCC*mec*. Such species might acquire the $mecA_{Sf}$ region from *S. fleurettii* to survive under conditions of antibiotic pressure, including exposure to fungi in the natural environment and medication in the livestock industry. Once SCC*mec* has been generated in or transferred into a broad-host-range *Staphylococcus* species, which could be isolated from various animals, as well as humans, the $mecA_{Sf}$ region carried by SCC*mec* might have spread beyond the host animal species. It is unknown whether SCC*mec* is generated in *S. aureus*, which is both a human-related and animal-related *Staphylococcus* species. It is our next agenda to find the bacterium in which SCC*mec* was generated as a mobile genetic element. One or more specific *Staphylococcus* species may play a role as a "reservoir" of the mobile genetic element SCC*mec*. Further studies are under way to investigate a reservoir of SCC*mec*.

We have been recently confronted with the rapid spread of community-acquired MRSA (27) and facilitated transmission of animal-derived MRSA to humans (6). It was previously considered that SCC*mec* is transmitted into *S. aureus* only rarely and that a limited number of MRSA clones have spread globally. However, new findings suggest that SCC*mec* is transmissible at least in a higher order of magnitude than originally suggested, that MRSA clones are emerging on numerous occasions in distinct locations, and that their geographic dispersals are limited (15). Besides promoting the efforts to restrict the use of antibiotics in livestock feeding, we also have to be vigilant against the dissemination of methicillin-resistant staphylococcal species across animal farms and human society.

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REFERENCES

1. **Antignac, A., and A. Tomasz.** 2009. Reconstruction of the phenotypes of methicillin-resistant *Staphylococcus aureus* by replacement of the staphylococcal cassette chromosome *mec* with a plasmid-borne copy of *Staphylococcus sciuri pbpD* gene. Antimicrob. Agents Chemother. **53:**435–441.
2. **Chandler, M., and J. Mahillon.** 2002. Insertion sequences revisited, p. 305–366. *In* N. L. Craig et al. (ed.), Mobile DNA II, 2nd ed. ASM Press, Washington, DC.
3. **Clinical and Laboratory Standards Institute.** 2009. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 8th ed., vol. 29, p. 2. Approved standard M7-A8. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Clinical and Laboratory Standards Institute.** 2009. Performance standards for antimicrobial susceptibility testing; 19th informational supplement M100-S19, vol. 29, p. 3. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Couto, I., H. de Lencastre, E. Severina, W. Kloos, J. A. Webster, R. J. Hubner, I. S. Sanches, and A. Tomasz.** 1996. Ubiquitous presence of a *mecA* homologue in natural isolates of *Staphylococcus sciuri*. Microb. Drug Resist. **2:**377–391.
6. **Goldburg, R., S. Roach, D. Wallinga, and M. Mellon.** 2008. The risks of pigging out on antibiotics. Science **321:**1294.
7. **Hanssen, A. M., and J. U. Ericson Sollid.** 2006. SCC*mec* in staphylococci: genes on the move. FEMS Immunol. Med. Microbiol. **46:**8–20.
8. **International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC).** 2009. Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements. Antimicrob. Agents Chemother. **53:**4961–4967.
9. **Ito, T., Y. Katayama, and K. Hiramatsu.** 1999. Cloning and nucleotide sequence determination of the entire *mec* DNA of pre-methicillin-resistant *Staphylococcus aureus* N315. Antimicrob. Agents Chemother. **43:**1449–1458.
10. **Jevons, M. P.** 1961. Celbenin-resistant staphylococci. Br. Med. J. **1:**124–125.
11. **Katayama, Y., T. Ito, and K. Hiramatsu.** 2000. A new class of genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **44:**1549–1555.
12. **Katayama, Y., T. Ito, and K. Hiramatsu.** 2001. Genetic organization of the chromosome region surrounding *mecA* in clinical staphylococcal strains: role of IS*431*-mediated *mecI* deletion in expression of resistance in *mecA*-carrying, low-level methicillin-resistant *Staphylococcus haemolyticus*. Antimicrob. Agents Chemother. **45:**1955–1963.
13. **Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu.** 2001. Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. Lancet **357:**1225–1240.
14. **Kwok, A. Y., and A. W. Chow.** 2003. Phylogenetic study of *Staphylococcus* and *Macrococcus* species based on partial *hsp60* gene sequences. Int. J. Syst. Evol. Microbiol. **53:**87–92.
15. **Nübel, U., P. Roumagnac, M. Feldkamp, J. H. Song, K. S. Ko, Y. C. Huang, G. Coombs, M. Ip, H. Westh, R. Skov, M. J. Struelens, R. V. Goering, B. Strommenger, A. Weller, W. Witte, and M. Achtman.** 2008. Frequent emergence and limited geographic dispersal of methicillin-resistant *Staphylococcus aureus*. Proc. Natl. Acad. Sci. U. S. A. **105:**14130–14135.
16. **Oliveira, D. C., S. W. Wu, and H. de Lencastre.** 2000. Genetic organization of the downstream region of the *mecA* element in methicillin-resistant *Staphylococcus aureus* isolates carrying different polymorphisms of this region. Antimicrob. Agents Chemother. **44:**1906–1910.
17. **Poyart, C., G. Quesne, C. Boumaila, and P. Trieu-Cuot.** 2001. Rapid and accurate species-level identification of coagulase-negative staphylococci by using the *sodA* gene as a target. J. Clin. Microbiol. **39:**4296–4301.
18. **Queck, S. Y., B. A. Khan, R. Wang, T. H. Bach, D. Kretschmer, L. Chen, B. N. Kreiswirth, A. Peschel, F. R. Deleo, and M. Otto.** 2009. Mobile genetic element-encoded cytolysin connects virulence to methicillin resistance in MRSA. PLoS Pathog. **5:**e1000533.
19. **Ryffel, C., R. Bucher, F. H. Kayser, and B. Berger-Bachi.** 1991. The *Staphylococcus aureus mec* determinant comprises an unusual cluster of direct repeats and codes for a gene product similar to the *Escherichia coli* sn-glycerophosphoryl diester phosphodiesterase. J. Bacteriol. **173:**7416–7422.
20. **Schleifer, K. H., U. Geyer, R. Kilpper-Bälz, and L. A. Devriese.** 1983. Elevation of *Staphylococcus sciuri* subsp. *lentus* (Kloos et al.) to species status: *Staphylococcus lentus* (Kloos et al.) comb. nov. Syst. Appl. Microbiol. **4:**382–387.
21. **Schmitz, F. J., M. Steiert, H. V. Tichy, B. Hofmann, J. Verhoef, H. P. Heinz, K. Kohrer, and M. E. Jones.** 1998. Typing of methicillin-resistant *Staphylococcus aureus* isolates from Dusseldorf by six genotypic methods. J. Med. Microbiol. **47:**341–351.
22. **Schnellmann, C., V. Gerber, A. Rossano, V. Jaquier, Y. Panchaud, M. G. Doherr, A. Thomann, R. Straub, and V. Perreten.** 2006. Presence of new *mecA* and *mph*(C) variants conferring antibiotic resistance in *Staphylococcus* spp. isolated from the skin of horses before and after clinic admission. J. Clin. Microbiol. **44:**4444–4454.
23. **Stepanovic, S., T. Hauschild, I. Dakic, Z. Al-Doori, M. Svabic-Vlahovic, L. Ranin, and D. Morrison.** 2006. Evaluation of phenotypic and molecular methods for detection of oxacillin resistance in members of the *Staphylococcus sciuri* group. J. Clin. Microbiol. **44:**934–937.
24. **Stranden, A., R. Frei, and A. F. Widmer.** 2003. Molecular typing of methicillin-resistant *Staphylococcus aureus*: can PCR replace pulsed-field gel electrophoresis? J. Clin. Microbiol. **41:**3181–3186.
25. **Svec, P., M. Vancanneyt, I. Sedlacek, K. Engelbeen, V. Stetina, J. Swings, and P. Petras.** 2004. Reclassification of *Staphylococcus pulvereri* Zakrzewska-Czerwinska et al. 1995 as a later synonym of *Staphylococcus vitulinus* Webster et al. 1994. Int. J. Syst. Evol. Microbiol. **54:**2213–2215.
26. **Takeda, Y., K. Takase, I. Yamato, and K. Abe.** 1998. Sequencing and characterization of the xyl operon of a gram-positive bacterium, *Tetragenococcus halophila*. Appl. Environ. Microbiol. **64:**2513–2519.
27. **Taubes, G.** 2008. The bacteria fight back. Science **321:**356–361.
28. **Tsubakishita, S., K. Kuwahara-Arai, T. Baba, and K. Hiramatsu.** 2010. Staphylococcal cassette chromosome *mec*-like element in *Macrococcus caseolyticus*. Antimicrob. Agents Chemother. **54:**1469–1475.
29. **Vernozy-Rozand, C., C. Mazuy, H. Meugnier, M. Bes, Y. Lasne, F. Fiedler, J. Etienne, and J. Freney.** 2000. *Staphylococcus fleurettii* sp. nov., isolated from goat's milk cheeses. Int. J. Syst. Evol. Microbiol. **50**(Pt. 4)**:**1521–1527.
30. **Webster, J. A., T. L. Bannerman, R. J. Hubner, D. N. Ballard, E. M. Cole, J. L. Bruce, F. Fiedler, K. Schubert, and W. E. Kloos.** 1994. Identification of the *Staphylococcus sciuri* species group with EcoRI fragments containing rRNA sequences and description of *Staphylococcus vitulus* sp. nov. Int. J. Syst. Bacteriol. **44:**454–460.
31. **Wilding, E. I., J. R. Brown, A. P. Bryant, A. F. Chalker, D. J. Holmes, K. A. Ingraham, S. Iordanescu, C. Y. So, M. Rosenberg, and M. N. Gwynn.** 2000. Identification, evolution, and essentiality of the mevalonate pathway for isopentenyl diphosphate biosynthesis in gram-positive cocci. J. Bacteriol. **182:**4319–4327.
32. **Wu, S., H. de Lencastre, and A. Tomasz.** 1998. Genetic organization of the *mecA* region in methicillin-susceptible and methicillin-resistant strains of *Staphylococcus sciuri*. J. Bacteriol. **180:**236–242.
33. **Wu, S. W., H. de Lencastre, and A. Tomasz.** 1999. The *Staphylococcus aureus* transposon Tn*551*: complete nucleotide sequence and transcriptional analysis of the expression of the erythromycin resistance gene. Microb. Drug Resist. **5:**1–7.