Clonal Complexes and Diversity of Exotoxin Gene Profiles in Methicillin-Resistant and Methicillin-Susceptible *Staphylococcus aureus* Isolates from Patients in a Spanish Hospital[▽]

M. A. Argudín,[1] M. C. Mendoza,[1] F. J. Méndez,[1,2] M. C. Martín,[3] B. Guerra,[4] and M. R. Rodicio[1]*

Área de Microbiología, Departamento de Biología Funcional, and Instituto Universitario de Biotecnología de Asturias, Universidad de Oviedo, Oviedo, Asturias, Spain[1]; Hospital Universitario Central de Asturias, Oviedo, Spain[2]; Instituto de Productos Lácteos de Asturias, Villaviciosa, Asturias, Spain[3]; and Federal Institute for Risk Assessment, Berlin, Germany[4]

Molecular epidemiology studies have allowed the identification of the methicillin (meticillin)-resistant (MRSA) and methicillin-susceptible (MSSA) clonal complexes (CCs) and clones of *Staphylococcus aureus* circulating in a Spanish hospital recently. Of 81 isolates tested, 32.1% were MRSA. Most of them carried staphylococcal cassette chromosome *mec* (SCC*mec*) IVc (88.5%) and belonged to CC5 (88.5%; multilocus sequence typing types ST125 [mainly associated with *spa* type t067], ST5, and ST228). A higher diversity was found among MSSA isolates (67.9%). Eighty percent shared the genetic background of major MRSA lineages (CC5 [38.2%; ST125 and ST5], CC30 [25.5%; ST30], CC45 [14.5%; ST45 and ST47], and CC8 [1.8%; ST8]), but CC12, CC15, CC51, and CC59 were also detected. Many exotoxin genes were present in each of the 81 isolates, independent of whether they were involved in sepsis (11 to 22) or other types of infections (13 to 21), and they appeared in 73 combinations. The relevant data are that (i) all isolates were positive for hemolysin and leukotoxin genes (98.8% for *lukED* and 25.9% for *lukPV*); (ii) all contained an enterotoxin gene cluster (*egc* with or without *seu*), frequently with one or more genes encoding classical enterotoxins; (iii) about half were positive for *tst* and 95% were positive for exfoliatin-encoding genes (*eta*, *etb*, and/or *etd*); and (iv) the four *agr* groups were detected, with *agrII* (55.6%) and *agrIII* (23.5%) being the most frequent. Taken together, results of the present study suggest a frequent acquisition and/or loss of exotoxin genes, which may be mediated by efficient intralineage transfer of mobile genetic elements and exotoxin genes therein and by eventual breakage of interlineage barriers.

Staphylococcus aureus is both a commensal bacterium and an extremely versatile pathogen that causes a wide range of diseases in humans, including superficial, deep-seated, and systemic infections, as well as a variety of toxemic syndromes, such as toxic shock syndrome (TSS), staphylococcal scalded-skin syndrome (SSSS), and staphylococcal food poisoning (36). *S. aureus* produces a wide range of virulence factors that mediate host colonization, invasion of damaged skin and mucosa, dissemination through the body, and evasion of host defense mechanisms (8, 12). Relevant among them are a variety of exotoxins that comprise α-, β-, γ-, and δ-hemolysins, leukotoxins (the classical LukS-PV–LukF-PV Panton-Valentine leukocidin [LukPV], LukE-LukD [LukED] and LukM-LukF′-PV [LukM]), exfoliative toxins, and pyrogenic toxin superantigens, such as the staphylococcal TSS toxin (TSST-1, first referred to as SEF) and staphylococcal enterotoxins (SEs) (14, 29, 43). Five major serological types of SEs, SEA through SEE (known as classical enterotoxins, encoded by the *sea* to *see* genes, respectively) have been initially identified. However, new types of SEs and their coding genes (*seg* through *seu*) were later reported. Several SE genes (*seg*, *sei*, *sem*, *sen*, and *seo*) are part of an operon termed the enterotoxin gene cluster (*egc*), of which a variant that contains *seu* instead of the two pseudogenes present in the originally described *egc* between *sei* and *sen* has been identified (27, 33). Both TSST-1 and SEs are potent activators of T-cell populations, leading to massive proliferation and uncontrolled release of proinflammatory cytokines (14). Expression of most virulence factors in *S. aureus* is under the control of the *agr* (accessory gene regulator) locus, which encodes a two-component signaling pathway and its activating ligand, a bacterial-density-sensing peptide termed the autoinducing peptide (37). *S. aureus* strains can be subdivided into four major *agr* groups, based on polymorphisms in the amino acid sequence of the autoinducing peptide and other components of the system (26, 28). Within a given group, each strain produces a peptide that can activate the *agr* response in other members of the group, whereas the autoinducing peptides produced by different groups are usually mutually inhibitory (26).

Apart from having pathogenic versatility, *S. aureus* can adapt rapidly to the selective pressure of antibiotics, with the emergence and spread of methicillin (meticillin)-resistant *S. aureus* (MRSA) isolates being a relevant example. Resistance to methicillin and other beta-lactam antibiotics is caused by the *mecA* gene, situated on a mobile genetic element, the *s*taphylococcal *c*assette *c*hromosome *mec* (SCC*mec*), which consists of the *mec* gene complex, the *ccr* gene complex, and the "junk-yard" regions. Based on the variability of the differently combined components, several types of SCC*mec* and several vari-

* Corresponding author. Mailing address: Área de Microbiología, Departamento de Biología Funcional, Facultad de Medicina, Julián Clavería 6, 33006 Oviedo, Spain. Phone: 34985103562. Fax: 34985103148. E-mail: rrodicio@fq.uniovi.es.

ants of the types have been distinguished (21, 23, 24, 25, 30, 31, 38, 45, 51).

In the present work, the techniques most commonly applied in epidemiological studies of *S. aureus* were used to identify the prevalent and sporadic MRSA and methicillin-susceptible *S. aureus* (MSSA) clones that have been causing disease in a Spanish hospital (the Hospital Universitario Central de Asturias [HUCA]) over a recent time period (2005 to 2006). These methods included pulsed-field gel electrophoresis (PFGE) of SmaI-digested genomic DNA (SmaI PFGE), *S. aureus* protein A gene (*spa*) typing, multilocus sequence typing (MLST) analysis, and SCC*mec* typing of MRSA (4, 9, 40, 42, 50, 52). The risk for human health posed by the accumulation of virulence genes in *S. aureus* (34) along with the potential application of such genes for subtyping prompted the assessment of the virulence gene repertoire of the HUCA isolates, with regard to the *agr* group and 30 exotoxin-encoding genes.

MATERIALS AND METHODS

S. aureus isolates. Eighty-one *S. aureus* isolates were analyzed in this study. Each was collected from a different patient attending the HUCA from December 2005 to December 2006. These isolates were identified in the hospital by use of standard procedures. Briefly, suspected colonies of *S. aureus* obtained in primary cultures were tested for agglutination with Pastorex Staph-plus (Bio-Rad Laboratories SA, Alcobendas, Madrid, Spain) and for thermonuclease (on DNase test agar; Biomedics, Madrid, Spain) and coagulase production (coagulase plasma; Becton Dickinson, San Agustín de Gualdalix, Madrid, Spain). Isolates that proved to be positive for the three tests were also evaluated for susceptibility or resistance to methicillin. *S. aureus* isolates were recovered from suppurative samples from spontaneous infections (5 isolates from patients with conjunctivitis, abscess in the hand, umbilical pyogenic granuloma, parotiditis, or infected ascites), surgical wounds (29 isolates associated with hand or leg amputations, hip, knee, or valvular prostheses, varicose veins, cellulitis, arthrodesis, osteomyelitis, Morton's neuroma, thoracotomy, tracheotomy, pleuritis [in two patients with esophageal cancer], or colon infection [in a patient with colon cancer]), urine (1 isolate from a patient with postcatheter urinary tract infection), tracheobronchial aspirates (11 isolates from patients with staphylococcal pneumonia, who have required intubation or assisted ventilation after cerebrovascular accident, brain injury, or polytraumatism), skin exudates (2 isolates from children with SSSS, 1 also involved in sepsis), and blood (33 isolates from patients who developed sepsis, most of them with severe underlying conditions, such as leukemia, stomach cancer, liver transplantation, cerebral vascular accident, thrombosis, aortic aneurysm, chronic renal insufficiency subjected to hemodialysis or peritoneal dialysis, osteomyelitis, pneumonia, and/or the presence of inserted catheters).

Macrorestriction-PFGE analysis. Whole DNA from each *S. aureus* isolate was analyzed by SmaI PFGE using a CHEF-DRIII SYS220/240 (Bio-Rad Laboratories, S.A., Madrid, Spain) system and the consensus protocol of the European HARMONY group (42). The resulting profiles were analyzed visually, and the presence or absence of fragments larger than ca. 20 kb was recorded. Those showing one or more mismatching bands were considered different and labeled with an "S" followed by a number. Genetic similarity between profiles was determined by the unweighted-pair method with arithmetic averages and Jaccard's coefficient, using the software program MVSP version 3.1 (Multivariate Statistics Package for PCs; RockWare Inc.). NCTC 8325 was used as a reference strain for PFGE.

***spa* typing and MLST.** *spa* typing and MLST were performed as described by Shopsin et al. (50) and Enright et al. (9), respectively. PCR products were sequenced by Qiagen (Hilden, Germany). *spa* types were identified with the software Ridom Staphtype (Ridom GmbH), and sequence types (STs) were assigned by using the MLST website (http://www.mlst.net). Isolates were grouped into a single clonal cluster (CC) when five of the seven housekeeping genes used in the MLST scheme were identical (9).

SCC*mec* typing and virulence gene content. SCC*mec* was typed by the multiplex PCR strategy reported by Zhang et al. (60). Primers for the exotoxin genes and the *agr* group have been described previously (3, 15, 16, 39, 41, 55). PCR assays were performed at least twice for each exotoxin gene and isolate, and positive and negative controls were always included. A dendrogram of similarity showing the clustering of the isolates according to virulence gene profiles was

constructed by using Jaccard's coefficient of similarity (BioNumerics version 5.1; Applied Maths).

Statistical methods. Statistical comparison between isolates giving positive and negative test results was performed with the χ^2 test, using SPSS software (version 15.0; SPSS, Chicago, IL). Differences between groups were considered statistically significant if P values were <0.05. The discrimination index (DI; i.e., the probability that two unrelated isolates would be assigned to different SmaI or virulence profiles) was calculated using Simpson's index of diversity (53).

RESULTS

Genotypic typing of the isolates. For a detailed assessment of the epidemiology of *S. aureus* circulating in the HUCA, the 81 isolates selected for the present study were subjected to SmaI PFGE, and representative subsets were also typed by *spa* typing and MLST.

SmaI PFGE yielded a DI of 0.98 and identified 50 profiles (S1 to S50; Fig. 1), with only 15 including more than one isolate (two up to seven). On the basis of the coefficient of similarity of Jaccard, a dendrogram was constructed (Fig. 2). At a cutoff point of 0.64, two major clusters (B and G), five minor clusters (A, C, D, E, and F), and several ungrouped branches were revealed. Cluster G, which accounted for 43.2% of the isolates, could be in turn separated into subclusters G1 and G2. Thirteen isolates of cluster G were selected for *spa* typing (Table 1). Four of them, all belonging to G1, were assigned to t002, another two were assigned to t3734, and seven were assigned to t067, and these last nine were included in G2. MLST performed on one or two isolates of each *spa* type associated t002 with ST5, while the t067 and t3734 isolates were associated with ST125 (which differs from ST5 by only one base pair in the amplified region of the *yqiL* gene [46]). Interestingly, representative isolates of cluster C and the single S20 isolate that generated the unclustered branch closest to cluster G were also of *spa* type t067 and ST125, whereas the next branch included three S25 isolates that were of *spa* type t109 and ST228 (a two-locus variant of ST5, with 1- and 2-bp differences in the *tpi* and *yqiL* amplified regions, respectively [48]). ST5, ST125, and ST228 belong to the same CC, namely, CC5, which clearly predominated in the HUCA.

With regard to the remaining SmaI PFGE clusters, the following observations were made. (i) Representative isolates of cluster B showed *spa* types t012 (four out of five tested) and t021 (the remaining one), and one isolate of each type proved to be of ST30 within CC30. (ii) Isolates belonging to cluster D were of *spa* types t282 and t1618 (one and two out of three tested, respectively), and one of the latter isolates was of ST45. (iii) Isolates in cluster A were of *spa* types t081, t383, and t1494, and the t383 isolate was of ST47. Both ST45 and ST47 (which differs from ST45 by one change in the amplified region of *aroE* [9]) belong to CC45, although the corresponding PFGE profiles grouped separately in the dendrogram. (iv) Two *spa* types and one ST were associated with clusters E (t547 and t084; both ST15) and F (t160 and t1381; ST12). Finally, the S32, S12, and S19 unclustered SmaI PFGE profiles were t024, t159, and nontypeable, respectively. The predicted ST for t024 is ST8 (CC8), t159 could be of ST121 or ST427 (CC51), and the last type was experimentally assigned to ST59 (CC59).

Incidence of MRSA and SCC*mec* typing. Of the isolates under study, 55 were MSSA (67.9%) and 26 were MRSA (32.1%). The latter belonged to SmaI PFGE clusters A, C, and
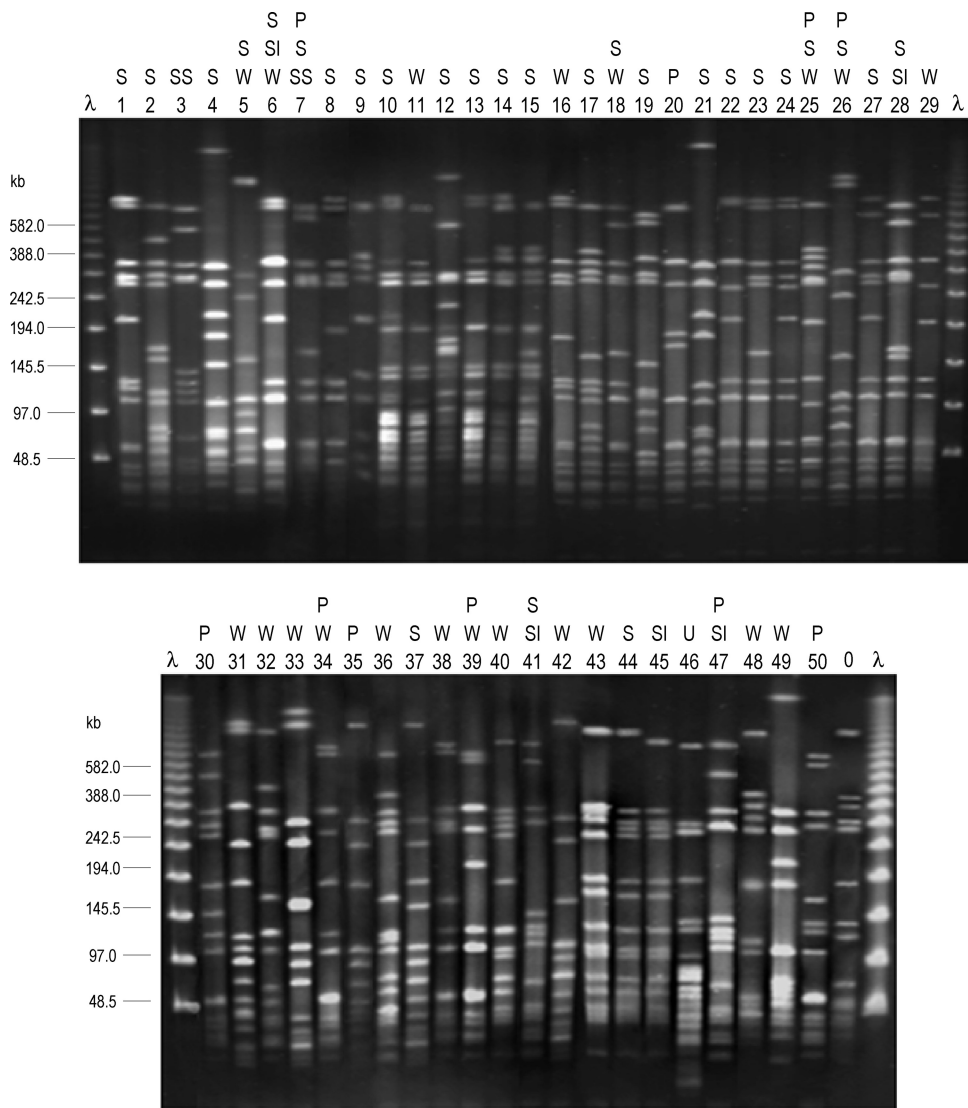
FIG. 1. SmaI PFGE analysis of *S. aureus* isolates from a Spanish hospital. Lanes λ, lambda ladder PFGE marker (New England Biolabs); lane 0, SmaI profile of strain NCTC 8325 included as a quality control; lanes 1 to 50, SmaI profiles generated from clinical isolates. P, pneumonia; S, sepsis; SI, spontaneous infection; SS, scalded skin syndrome; U, urinary tract infection; W, surgical wound infection. This is a composite figure from five gels that were run under identical PFGE conditions.

G or generated one of three unclustered patterns (S20, S25, and S48). However, more than half (16 out of 26) grouped in subcluster G2 (*spa* type t067 or t3734; ST125) within cluster G, and nearly all were members of CC5 (Fig. 2). All MRSA isolates contained SCC*mec* IVc, except two that carried SCC*mec* I and generated the unclustered S25 profile (*spa* type t109; ST228) and one that was nontypeable by the applied method (t067; subcluster G2).

Virulence gene profiles. The *agr* group and the exotoxin gene repertoire were determined for the 81 *S. aureus* isolates of the HUCA and for *S. aureus* NCTC 8325 (included as a control). A dendrogram of similarity constructed on the basis of the presence or absence of the screened genes separated the control strain from the HUCA isolates and revealed a wide heterogeneity among the latter (Fig. 3). In fact, they were distributed into 73 virulence gene profiles, with only six being shared

by more than one isolate (two or three). Relevant data were as follows. (i) The four types of *agr* systems were represented in the series, with *agrII* being the most common (55.6%), followed by *agrIII* (23.5%), *agrIV* (12.3%), and *agrI* (8.6%). (ii) All isolates were beta-hemolytic and positive for *hla* and *hlg-2* (encoding α- and γ-variant hemolysins, respectively). A clearly prevalent profile (92.6%) included the five hemolysin genes tested (*hla–hlb–hld–hlg–hlg-2*). (iii) All isolates carried at least one leukotoxin-encoding gene, most frequently *lukED* (98.8%), but *lukPV* also occurred (25.9%). In contrast, *lukM* was not detected. (iv) A remarkable number of isolates (95.1%) was positive for genes encoding exfoliative toxins. *etb* (89.9%), found alone or in combination with *eta* and/or *etd*, was the most common. It was followed by *eta* and *etd*, carried by 43.2% and 17.3% of the isolates, respectively. Despite this, only two young children were diagnosed with SSSS, with one of
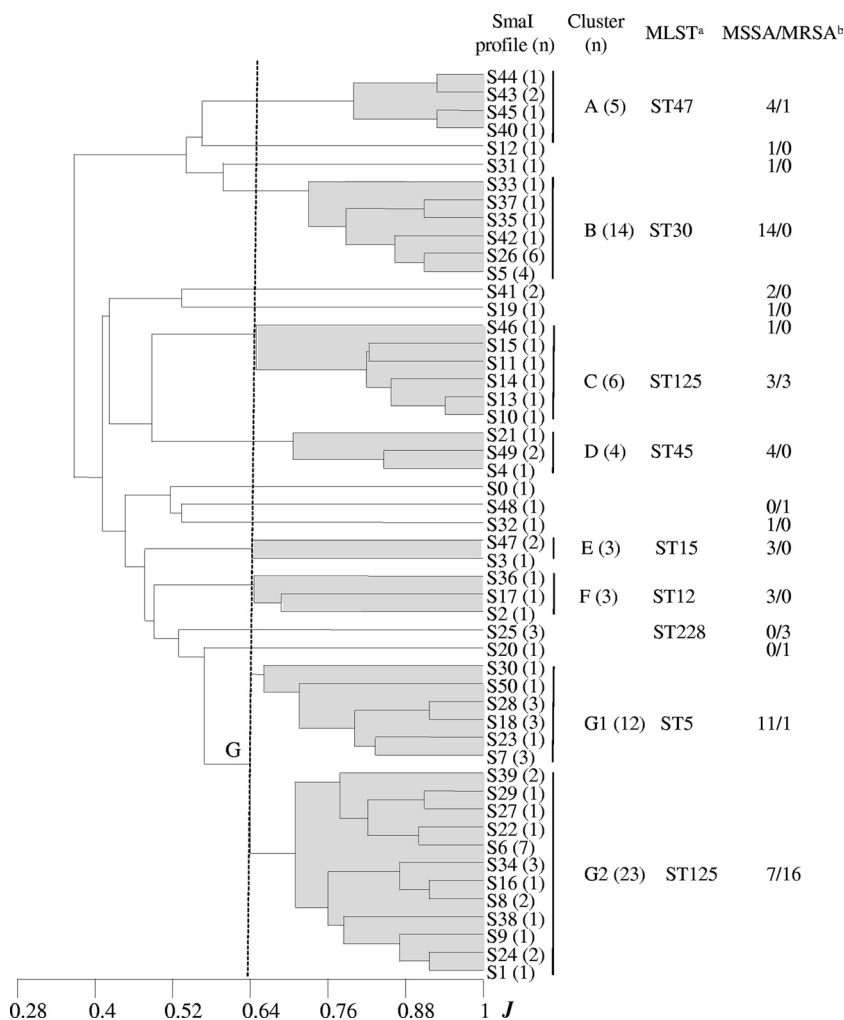
FIG. 2. Dendrogram showing the relatedness between SmaI macrorestriction fragment profiles generated from the *S. aureus* clinical isolates and the control strain (NCTC 8325). At a Jaccard coefficient of similarity (*J*) of 0.64, seven clusters (labeled A to G, the latter with subclusters G1 and G2) and several unclustered branches were detected. Footnote a, determined multilocus sequence types of representative isolates; footnote b, MSSA and MRSA. n, number of isolates.

TABLE 1. Relationships between CC, MLST type, *spa* type, and PFGE cluster[a]

CC	MLST type(s) (n)[b]	*spa* type(s) (n)[c]	PFGE cluster[d]
CC5	ST5 (2)	t002 (4)	G1
	ST125 (2)	t067 (7), t3734 (2)	G2
	ST125	t067 (4)	C
	ST228	t109 (3)	–
	(ST125)	t067	–
CC8	(ST8)	t024	–
CC12	ST12	t160, t1381	F
CC15	ST15 (2)	t084 (2), t547	E
CC30	ST30 (2)	t012 (4), t021	B
CC45	ST45	t282, t1618 (2)	D
	ST47	t081 (2), t383, t1494	A
CC51	(ST121, ST427)	t159	–
CC59	ST59	NT	–

[a] The numbers of isolates (n) are listed in parentheses if the number is more than one.
[b] Determined or presumptive (according to the *spa* type; in parentheses) STs of representative isolates are shown.
[c] NT, nontypeable.
[d] –, unclustered PFGE profile.

the responsible isolates being positive for *etb*, *eta*, and *agrIV* and the other being positive for *etb* and *agrII*. (v) About half of the isolates (51.9%) carried *tst*, although none of the patients suffered from TSS. (vi) All isolates contained an *egc* or an *egc*-like cluster (70.4% and 29.6%, respectively), frequently together with other SE genes, including those encoding classical enterotoxins (92.6%). Of the latter, *sec*, *sea*, *seb*, and *sed* were present in 80.2%, 55.6%, 27.2%, and 24.7% of the isolates, respectively. Each of these genes appeared either alone (in a few cases) or with one or two of the other in different combinations. All HUCA isolates were negative for *see*. The *sed*, *sej*, and *ser* genes carried by plasmids were present in 24.7% of the isolates, with the three coinciding in 17.3% of them. Among other SE genes, *sep* was the most frequent (24.7%), but *seh*, *sek*, *sel*, and *seq* were also represented (12.3%, 2.5%, 2.5%, and 3.7%, respectively). Interestingly, typing of the isolates on the basis of the virulence gene profile yielded a DI close to 1 (0.997).

Overall, a very high number of exotoxin genes was present in

FIG. 3. Dendrogram showing the relatedness between virulence gene profiles (for 30 exotoxin genes and the *agr* group) of the *S. aureus* isolates tested. At a Jaccard coefficient of similarity (*J*) of ca. 0.63, two main clusters, termed V1 and V2, and three subclusters within V2 (V2a, V2b, and V2c) are indicated. The type of infection caused by each isolate, the SmaI PFGE cluster to which it belongs, the SCC*mec* type (I, IVc, or not typeable [nt]) of MRSA, and the *spa* type as well as the MLST type of representative isolates, are shown at the right of the dendrogram.

TABLE 2. Characteristics of the virulence clusters and subclusters[a]

Virulence cluster (n)	agr type (n)	CC (n)	Cytotoxin(s) (n)[b]	Pyrogenic toxin superantigen gene(s) (n)[b]
V1	*agrI* (4)	CC8, CC30, CC45	**lukED** (4), *lukPV* **eta** (3), **etb** (4), *etd* (2)	*tst* (2), *sea*, *seb* (2), **sec** (4) **sed** (4), **sej** (3), **ser** (3) *seh* **egc** (3), *egc*-like
	agrII (12)	**CC5** (9), CC15, CC45	**lukED** (12), *lukPV* (2) *eta* (6), **etb** (11), *etd* (3)	*tst* (3), *sea* (7), *seb* (4), **sec** (10) **sed** (12), **sej** (12), **ser** (10) *seh* (3), *sep* (3) **egc** (9), *egc*-like (3)
	agrIV (3)	CC5, CC45	**lukED** (3), *lukPV* *eta*, *etb* (2), *etd*	*tst* (2), *sea* (2), *seb*, **sec** (3) **sed** (3), **sej** (3), **ser** (2) *sek*, *sel*, *sep*, *seq* *egc* (2), *egc*-like
V2a (5)	*agrIV* (5)	**CC5** (4), CC45	**lukED** (4), **lukPV** (5) **eta** (5), **etb** (5), *etd*	**tst** (5), **sea** (5), **sec** (4) *sel* *egc*, **egc-like** (4)
V2b (16)	*agrIII* (16)	CC5 (4), **CC30** (11)	**lukED** (16), *lukPV* (7) *eta* (6), **etb** (15)	**tst** (15), **sea** (15), *seb*, **sec** (14) *sed*, *sej* *seh* (4) *egc* (4), **egc-like** (12)
V2c (34)	*agrII* (32)	**CC5** (28), CC15, CC45	**lukED** (32), *lukPV* (5) *eta* (12), **etb** (28), *etd* (2)	*tst* (10), *sea* (13), *seb* (11), **sec** (25) *seh* (2), *sep* (14), *seq* **egc** (30), *egc*-like (2)
	agrIV	CC5	*lukED*, *etb*	*sec*, *sep* *egc*
	agrI	CC45	*lukED*, *etb*, *etd*	*tst*, *sea*, *sec* *egc*

[a] The numbers of isolates (n) are listed in parentheses if the number is more than one.
[b] All isolates carried three to five hemolysin genes; exotoxin genes detected in all or most (more than 75%) isolates of the corresponding virulence cluster or subcluster are shown in boldface.

each of the HUCA isolates, independently of whether they were involved in spontaneous infections (13 up to 16), surgical wound infections (13 up to 21), pneumonia (13 up to 19), sepsis (11 up to 22), or SSSS (followed or not followed by sepsis, 14 and 18, respectively). Among the screened genes, only *sep* was significantly more frequent in isolates associated with sepsis ($P = 0.032$), and *seb* was more frequent in those that caused other infections ($P = 0.032$). Moreover, *agrII* and *sep* were more common in MRSA isolates than in MSSA isolates (P values of 0.029 and 0.048, respectively), while *agrIII* and *tst* were more common in MSSA isolates (P values of 0.021 and 0.032, respectively).

At a Jaccard coefficient of ca. 0.63, the dendrogram shown in Fig. 3 separated the HUCA isolates into two main virulence clusters (termed V1 and V2; Table 2). V1 incorporated all except one of the isolates that carried enterotoxin genes from plasmids (*sed*, *sej*, and/or *ser*), which were scattered among three of the four *agr* groups (*agrII*, *agrI*, and *agrIV*, with 12, 4, and 3 isolates, respectively). Most V2 isolates fell within one of three subclusters (V2a, V2b, and V2c). V2a included five *agrIV* isolates, all positive for *lukPV*, *eta*, *etb*, *tst*, *sea*, and the five hemolysin genes tested. With single exceptions, *lukED*, *sec*, and *egc*-like genes were also present. V2b grouped most of the *agrIII* isolates (16 out of 19) characterized by the occurrence of the five hemolysin genes, *lukED* (all isolates), *etb*, *tst*, *sea* (each with a single exception), and *sec* (two exceptions). In addition, most carried the *egc*-like cluster, and nearly half tested positive for *lukPV*. V2c included most of the *agrII* isolates which lacked SE genes from plasmids. The hemolysin genes, *lukED*, *etb*, *sec*, and *egc* were detected in all or most isolates of the subcluster

(from ca. 80% up to 100%), while *eta*, *tst*, *sea*, *seb*, and *sep* were relatively common (32.4% to 44.1%). Finally, data compiled in Fig. 3 failed to reveal a correlation between virulence gene profile(s) and type of disease, with all clusters and subclusters grouping isolates involved in sepsis together with isolates that caused other types of infections. Accordingly, virulence factors apart from those screened in the present work, differential expression of the detected genes, and/or host-related factors may have been critical for the outcome of the disease (32, 35, 58).

DISCUSSION

Molecular epidemiology studies allowed the identification of the MRSA and MSSA CCs and clones that have been circulating in the HUCA during a recent time period. Overall, the frequency of MRSA isolates in this hospital (32.1%) coincided with that reported by the EPINE group, which surveys the prevalence of nosocomial infections in Spain (32.5% for the 2001-to-2003 period [2]), and was also in line with the results of a single-day surveillance study that analyzed 439 *S. aureus* isolates recovered from 143 Spanish hospitals during 2002 (30.5%) (5).

Most MRSA isolates from the HUCA (73%) were ST125-SCC*mec* IV isolates. This clone emerged in Spain during 1996, although it was first reported in 2001, and has now become predominant in Spanish hospitals (46, 47, 56). At a lower frequency, it was also found in Norway (13). As in the present study, a strong correlation between ST125 and *spa* type t067 was observed in both countries. Most of the remaining MRSA

isolates from the HUCA were also positive for SCC*mec* IV but belonged to other STs (i.e., ST5, ST228, or ST47); two carried SCC*mec* I and were of ST228, while one was not typeable by the applied method (60). The ST5-SCC*mec* IV (pediatric clone) (49) and ST228-SCC*mec* I (southern German clone) (48) clones are major hospital-acquired MRSA clones, widely distributed worldwide (7, 46, 56). In summary, typing of MRSA isolates from the HUCA revealed that nearly all belonged to CC5 (88.5%), one of the most diversified lineages of MRSA (48), and carried SCC*mec* IV (also 88.5%), specifically SCC*mec* IVc, which in Spain accounted only for 24% of the MRSA isolates, whereas subtype IVa was the most frequent (73.3%) (47).

As previously reported by other authors (7, 10, 52), a higher diversity of CCs was found among the MSSA isolates of the HUCA, which accounted for 67.9% of the analyzed isolates. Of them, 80.0% had a genetic background common to major MRSA lineages, namely, CC5 (38.2%), CC30 (25.5%), CC45 (14.5%), and CC8 (1.8%) (7, 17, 48). As was the case for MRSA, MSSA isolates with a CC5 genetic background mostly belonged to SmaI PFGE subclusters G1 and G2 and were of either ST5 or ST125; CC30 isolates fell into SmaI PFGE cluster B and were of ST30; and ST47 and ST45, both members of CC45, were separated by SmaI PFGE into clusters A and D. Four successful MSSA lineages different from the major MRSA clones, namely, CC12, CC15 (PFGE clusters F and E), CC51, and CC59 (each with one sporadic isolate), were also detected. Isolates of these four lineages have been observed in both nosocomial and community settings in different countries (1, 18, 19, 44, 54, 57). Finally, it is worth noting that, like in other studies (4, 20), a good concordance between typing by SmaI PFGE, *spa* type, and MLST type was found for the HUCA isolates. In fact, most isolates with the same MLST had similar SmaI profiles and, hence, grouped within the same PFGE cluster. However, several SmaI PFGE clusters and/or branches could be associated with a certain CC and even with a certain ST, consistent with the higher discrimination of the former method.

With regard to the virulence gene repertoires, the high number of exotoxin genes carried by each of the 81 isolates analyzed in the present work (from 11 up to 22) is remarkable. All HUCA isolates harbored genes encoding four or five hemolysins, one or two leukocidins, and five up to 13 enterotoxins. All tested positive for *egc* with or without *seu*, and most were positive for one or more genes encoding classical enterotoxins, including high percentages of *sec* and *sea*. Moreover, all except four isolates contained at least one exfoliatin gene (*eta*, *etb*, and/or *etd*), whereas *tst* was present in half of them, and the leukotoxin gene *lukPV* was present in about one-fourth. In Spanish hospitals, the incidence of MSSA isolates carrying *lukPV* has increased from 0% in 2002 up to 36.4% in 2006 to 2007 (5, 47). In the present study, 66.7% of the *lukPV*-positive isolates were MSSA, but 33.3% were MRSA, and most belonged to the prevalent CC5. Of the 30 virulence genes screened, only *lukM* and *see* were absent from the series. Those that were detected appeared in many different combinations, with a total of 73 virulence profiles displayed by 81 isolates. A wide diversity of virulence gene combinations has also been reported for *S. aureus* isolates from England, Germany, and France (6, 22, 35).

Overall, the high number of exotoxin genes carried by each isolate and the rather indiscriminate distribution of these genes among different *agr* groups and genomic backgrounds, together with the striking diversity of exotoxin gene combinations, are consistent with a frequent acquisition and/or loss of the screened genes. It is widely accepted that mobile genetic elements carrying exotoxin genes can efficiently spread within lineages (35). In addition, they may be eventually capable of breaking interlineage barriers. An important barrier is the SauI type I restriction-modification system, which appears to be one of the major mechanisms underlying the clonal structure of *S. aureus* (11, 59). Nevertheless, a mobile genetic element can enter a CC from which it was originally excluded by means of a SauI restriction mutant. Once in such a mutant, the element might become modified at the specific sequences recognized by the *hsdS1* and *hsdS2* products characteristic of this particular lineage. Afterwards, its transfer to other members of the same lineage would be possible. In any case, the accumulation of exotoxin genes within *S. aureus* isolates is a matter of concern, since it may result in enhanced pathogenicity under appropriate circumstances (34). In the HUCA, the epidemiological surveillance of these potential "superbugs" could be facilitated, at least in the near future, by these findings with regard to the remarkable diversity of exotoxin gene combinations, which nearly differentiated at the strain level.

REFERENCES

1. **Aires de Sousa, M., T. Conceição, C. Simas, and H. de Lencastre.** 2005. Comparison of genetic backgrounds of methicillin-resistant and -susceptible *Staphylococcus aureus* isolates from Portuguese hospitals and the community. J. Clin. Microbiol. **43:**5150–5157.
2. **Asensio, A., R. Canton, J. Vaque, J. Rossello, F. Calbo, J. Garcia-Caballero, V. Dominguez, A. Hernandez, A. Trilla, and EPINE Working Group.** 2006. Nosocomial and community-acquired methicillin-resistant *Staphylococcus aureus* infections in hospitalized patients (Spain, 1993–2003). J. Hosp. Infect. **63:**465–471.
3. **Becker, K., A. W. Friedrich, G. Lubritz, M. Weilert, G. Peters, and C. Von Eiff.** 2003. Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. J. Clin. Microbiol. **41:**1434–1439.
4. **Cookson, B. D., D. A. Robinson, A. B. Monk, S. Murchan, A. Deplano, R. de Ryck, M. J. Struelens, C. Scheel, V. Fussing, S. Salmenlinna, J. Vuopio-Varkila, C. Cuny, W. Witte, P. T. Tassios, N. J. Legakis, W. van Leeuwen, A. van Belkum, A. Vindel, J. Garaizar, S. Haeggman, B. Olsson-Liljequist, U. Ransjo, M. Muller-Premru, W. Hryniewicz, A. Rossney, B. O'Connell, B. D. Short, J. Thomas, S. O'Hanlon, and M. C. Enright.** 2007. Evaluation of molecular typing methods in characterizing a European collection of epidemic methicillin-resistant *Staphylococcus aureus* strains: the HARMONY collection. J. Clin. Microbiol. **45:**1830–1837.
5. **Cuevas, O., E. Cercenado, E. Bouza, C. Castellares, P. Trincado, R. Cabrera, A. Vindel, and Spanish Group for the Study of *Staphylococcus*.** 2007. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in Spain: a multicentre prevalence study (2002). Clin. Microbiol. Infect. **13:**250–256.
6. **Dauwalder, O., G. Lina, G. Durand, M. Bes, H. Meugnier, V. Jarlier, B.**

Coignard, F. Vandenesch, J. Etiene, and F. Laurent. 2008. Epidemiology of invasive methicillin-resistant *Staphylococcus aureus* clones collected in France in 2006 and 2007. J. Clin. Microbiol. **46:**3454–3458.

7. **Deurenberg, R. H., and E. E. Stobbering.** 2008. The evolution of *Staphylococcus aureus*. Infect. Genet. Evol. **8:**747–763.

8. **Dinges, M. M., P. M. Orwin, and M. Schlievert.** 2000. Exotoxins of *Staphylococcus aureus*. Clin. Microbiol. Rev. **13:**16–34.

9. **Enright, M. C., N. P. Day, C. E. Davis, S. J. Peacock, and B. G. Spratt.** 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J. Clin. Microbiol. **38:**1008–1015.

10. **Faria, N. A., J. A. Carrico, D. C. Oliveira, M. Ramirez, and H. de Lencastre.** 2008. Analysis of typing methods for epidemiological surveillance of both methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains. J. Clin. Microbiol. **46:**136–144.

11. **Feng, Y., C.-J. Chen, L.-H. Su, S. Hu, J. Yu, and C.-H. Chiu.** 2008. Evolution and pathogenesis of *Staphylococcus aureus*: lessons learned from genotyping and comparative genomics. FEMS Microbiol. Rev. **32:**23–37.

12. **Ferry, T., T. Perpoint, F. Vandenesch, and J. Etienne.** 2005. Virulence determinants in *Staphylococcus aureus* and their involvement in clinical syndromes. Curr. Infect. Dis. Rep. **7:**420–428.

13. **Fossum, A. E., and G. Butcholm.** 2006. Increased incidence of methicillin-resistant *Staphylococcus aureus* ST80, novel ST125 and SCC*mec*IV in the south-eastern part of Norway during a 12-year period. Clin. Microbiol. Infect. **12:**627–633.

14. **Fraser, J. D., and T. Proft.** 2008. The bacterial superantigen and superantigen-like proteins. Inmunol. Rev. **225:**226–243.

15. **Fueyo, J. M., M. C. Mendoza, M. A. Alvarez, and M. C. Martin.** 2005. Relationships between toxin gene content and genetic background in nasal carried isolates of *Staphylococcus aureus* from Asturias, Spain. FEMS Microbiol. Lett. **243:**447–454.

16. **Fueyo, J. M., M. C. Mendoza, M. R. Rodicio, J. Muñiz, M. A. Alvarez, and M. C. Martin.** 2005. Cytotoxin and pyrogenic toxin superantigen gene profiles of *Staphylococcus aureus* associated with subclinical mastitis in dairy cows and relationships with macrorestriction genomic profiles. J. Clin. Microbiol. **43:**1278–1284.

17. **Gomes, A. R., H. Westh, and H. de Lencastre.** 2006. Origins and evolution of methicillin-resistant *Staphylococcus aureus* clonal lineages. Antimicrob. Agents Chemother. **50:**3237–3244.

18. **Grundmann, H., S. Hori, M. C. Enright, C. Webster, A. Tami, E. J. Feil, and T. Pitt.** 2002. Determining the genetic structure of the natural population of *Staphylococcus aureus*: a comparison of multilocus sequence typing with pulsed-field gel electrophoresis, randomly amplified polymorphic DNA analysis, and phage typing. J. Clin. Microbiol. **40:**4544–4546.

19. **Hallin, M., O. Denis, A. Deplano, R. De Mendonça, R. De Ryck, S. Rottiers, and M. J. Struelens.** 2007. Genetic relatedness between methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*: results of a national survey. J. Antimicrob. Chemother. **59:**465–472.

20. **Hallin, M., A. Deplano, O. Denis, R. De Mendonça, R. De Ryck, and M. J. Strudels.** 2007. Validation of pulsed-field gel electrophoresis and *spa* typing for long-term, nationwide epidemiological surveillance studies of *Staphylococcus aureus* infections. J. Clin. Microbiol. **45:**127–133.

21. **Hisata, K., K. Kuwahara-Arai, M. Yamanoto, T. Ito, Y. Nakatomi, L. Cui, T. Baba, M. Terasawa, C. Sotozono, S. Kinoshita, Y. Yamashiro, and K. Hiramatsu.** 2005. Dissemination of methicillin-resistant staphylococci among healthy Japanese children. J. Clin. Microbiol. **43:**3364–3372.

22. **Holtfreter, S., D. Grumann, M. Schmudde, H. T. T. Nguyen, P. Eichler, B. Strommenger, K. Kopron, J. Kotola, S. Giedrys-Kalemba, I. Steinmetz, W. White, and B. M. Bröker.** 2007. Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. J. Clin. Microbiol. **45:**2669–2680.

23. **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.

24. **Ito, T., Y. Katayama, K. Asada, N. Mori, K. Tsutsumimoto, C. Tiensasitorn, and K. Hiramatsu.** 2001. Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome of MRSA strains in the world. Antimicrob. Agents Chemother. **45:**1323–1336.

25. **Ito, T., X. X. Ma, F. Takeuchi, K. Okuma, H. Yuzawa, and K. Hiramatsu.** 2004. Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *ccrC*. Antimicrob. Agents Chemother. **48:**2637–2651.

26. **Jarrraud, S., G. J. Lyon, A. M. Figueiredo, G. Lina, F. Vandenesch, J. Etienne, T. W. Muir, and R. P. Novick.** 2000. Exfoliatin-producing strains define a fourth *agr* specificity group in *Staphylococcus aureus*. J. Bacteriol. **182:**6517–6522.

27. **Jarrraud, S., M. A. Peyrat, A. Lim, A. Tristan, M. Bes, C. Mougel, J. Etienne, F. Vandenesch, M. Bonneville, and G. Lina.** 2001. *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. J. Immunol. **166:**669–677.

28. **Ji, G., R. Beavis, and R. P. Novick.** 1997. Bacterial interference caused by autoinducing peptide variants. Science **276:**2027–2030.

29. **Kaneko, J., and Y. Kamio.** 2004. Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. Biosci. Biotechnol. Biochem. **68:**981–1003.

30. **Katayama, Y., T. Ito, and K. Hiramatsu.** 2000. A new class of genetic element, staphylococcal cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **44:**1549–1555.

31. **Kwon, N. H., K. T. Park, J. S. Moon, W. K. Jung, S. H. Kim, J. M. Kim, S. K. Hong, H. C. Koo, Y. S. Joo, and Y. H. Park.** 2005. Staphylococcal cassette chromosome *mec* (SCC*mec*) characterization and molecular analysis for methicillin-resistant *Staphylococcus aureus* and novel SCC*mec* subtype IVg isolated from bovine milk in Korea. J. Antimicrob. Chemother. **56:**624–632.

32. **Labandeira-Rey, M., F. Couzon, S. Boisset, E. L. Brown, M. Bes, Y. Benito, E. M. Barbu, V. Vazquez, M. Höök, J. Etienne, F. Vandenesch, and M. G. Bowden.** 2007. *Staphylococcus aureus* Panton-Valentine leukocidin causes necrotizing pneumonia. Science **315:**1130–1133.

33. **Letertre, C., S. Perelle, F. Dilasser, and P. Fach.** 2003. Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus*. J. Appl. Microbiol. **95:**38–43.

34. **Lindsay, J. A., and M. T. G. Holden.** 2004. *Staphylococcus aureus*: superbug, super genome? Trends Microbiol. **12:**378–385.

35. **Lindsay, J. A., C. E. Moore, N. P. Day, S. J. Peacock, A. A. Witney, R. A. Stabler, S. E. Husain, P. D. Butcher, and J. Hinds.** 2006. Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. J. Bacteriol. **188:**669–676.

36. **Lowy, F. D.** 1998. *Staphylococcus aureus* infections. N. Engl. J. Med. **339:**520–532.

37. **Lyon, G. J., and R. P. Novick.** 2004. Peptide signaling in *Staphylococcus aureus* and other Gram-positive bacteria. Peptides **25:**1389–1403.

38. **Ma, X. X., T. Ito, C. Tiensasitorn, M. Jamklang, P. Chongtrakool, S. Boyle-Vavra, R. S. Daum, and K. Hiramatsu.** 2002. Novel type of staphylococcal cassette chromosome *mec* identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. Antimicrob. Agents Chemother. **46:**1147–1152.

39. **McClure, J. A., J. M. Conly, V. Lau, S. Elsayed, T. Louie, W. Hutchins, and K. Zhang.** 2006. Novel multiplex PCR assay for detection of the staphylococcal virulence marker Panton-Valentine leukocidin genes and simultaneous discrimination of methicillin-susceptible from -resistant staphylococci. J. Clin. Microbiol. **44:**1141–1144.

40. **McDougal, L. K., C. D. Steward, G. E. Killgore, J. M. Chaitram, S. K. McAllister, and F. C. Tenover.** 2003. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J. Clin. Microbiol. **41:**5113–5120.

41. **Moore, P. C. L., and J. A. Lindsay.** 2001. Genetic variation among hospital isolates of methicillin-sensitive *Staphylococcus aureus*: evidence of horizontal transfer of virulence genes. J. Clin. Microbiol. **39:**2760–2767.

42. **Murchan, S., M. E. Kaufmann, A. Deplano, R. de Ryck, M. Struelens, C. E. Zinn, V. Fussing, S. Salmenlinna, J. Vuopio-Varkila, N. El Solh, C. Cuny, W. Witte, P. T. Tassios, N. Legakis, W. van Leeuwen, A. van Belkum, A. Vindel, I. Laconcha, J. Garaizar, S. Haeggman, B. Olsson-Liljequist, U. Ransjo, G. Coombes, and B. Cookson.** 2003. Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. J. Clin. Microbiol. **41:**1574–1585.

43. **Nishifuji, K., M. Sugai, and M. Amagai.** 2008. Staphylococcal exfoliative toxins: "molecular scissors" of bacteria that attack the cutaneous defense barrier in mammals. J. Dermatol. Sci. **49:**21–31.

44. **Nulens, E., E. E. Stobberingh, H. van Dessel, S. Sebastian, F. H. van Tiel, P. B. Beisser, and R. H. Deurenberg.** 2008. Molecular characterization of *Staphylococcus aureus* bloodstream isolates collected in a Dutch university hospital between 1999 and 2006. J. Clin. Microbiol. **46:**2438–2441.

45. **Oliveira, D. C., A. Tomasz, and H. de Lencastre.** 2001. The evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*: identification of two ancestral genetic backgrounds and the associated *mec* elements. Microb. Drug Resist. **7:**349–361.

46. **Pérez-Roth, E., F. Lorenzo-Díaz, N. Batista, A. Moreno, and S. Méndez-Alvarez.** 2004. Tracking methicillin-resistant *Staphylococcus aureus* clones during a 5-year period (1998 to 2002) in a Spanish hospital. J. Clin. Microbiol. **42:**4649–4656.

47. **Pérez-Vázquez, M., A. Vindel, C. Marcos, J. Oteo, O. Cuevas, P. Trincado, V. Bautista, H. Grundmann, J. Campos, et al.** 2009. Spread of invasive Spanish *Staphylococcus aureus* *spa*-type t067 associated with a high prevalence of aminoglycoside-modifying enzyme *ant(4')-Ia* and the efflux pump genes *mrsA/mrsB*. J. Antimicrob. Chem. **63:**21–31.

48. **Robinson, D. A., and M. C. Enright.** 2003. Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **47:**3926–3934.

49. **Sá-Leão, R., I. Santos Sanches, D. Dias, I. Peres, R. M. Barros, and H. de Lencastre.** 1999. Detection of an archaic clone of *Staphylococcus aureus* with low-level resistance to methicillin in a pediatric hospital in Portugal and in

international samples: relics of a formerly widely disseminated strain? J. Clin. Microbiol. **37:**1913–1920.

50. **Shopsin, B., M. Gomez, S. O. Montgomery, D. H. Smith, M. Waddington, D. E. Dodge, D. A. Bost, M. Riehman, S. Naidich, and B. N. Kreiswirth.** 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J. Clin. Microbiol. **37:**3556–3563.

51. **Shore, A., A. S. Rossney, C. T. Keane, M. C. Enright, and D. C. Coleman.** 2005. Seven novel variants of the staphylococcal chromosomal cassette *mec* in methicillin-resistant *Staphylococcus aureus* isolates from Ireland. Antimicrob. Agents Chemother. **49:**2070–2083.

52. **Strommenger, B., C. Braulke, D. Heuck, C. Schmidt, B. Pasemann, U. Nubel, and W. Witte.** 2008. *spa* typing of *Staphylococcus aureus* as a frontline tool in epidemiological typing. J. Clin. Microbiol. **46:**574–581.

53. **Struelens, M. J., A. Bauernfeind, A. Van Belkum, D. Blanc, B. D. Cookson, L. Dijkshoorn, N. El Solh, J. Etienne, J. Garaizar, P. Gerner-Smidh, N. Legakis, H. de Lencastre, M. H. Nicolas, T. L. Pitt, U. Römling, V. Rosdahl, and W. Witte.** 1996. Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. Clin. Microbiol. Infect. **2:**2–11.

54. **Takano, T., W. Higuchi, T. Otsuka, T. Baranovich, S. Enany, K. Saito, H. Isobe, S. Dohmae, K. Ozaki, M. Takano, Y. Iwao, M. Shibuya, T. Okubo, S. Yabe, D. Shi, I. Reva, L.-J. Teng, and T. Yamamoto.** 2008. Novel characteristics of community-acquired methicillin-resistant *Staphylococcus aureus* strains belonging to multilocus sequence type 59 in Taiwan. Antimicrob. Agents Chemother. **52:**837–845.

55. **Tenover, F. C., L. K. McDougal, R. V. Goering, G. Killgore, S. J. Projan, J. B.**

Patel, and P. M. Dunman. 2006. Characterization of a strain of community-associated methicillin-resistant *Staphylococcus aureus* widely disseminated in the United States. J. Clin. Microbiol. **44:**108–118.

56. **Vindel, A., P. Trincado, E. Gómez, R. Cabrera, T. Boquete, C. Solá, S. Valdezate, and J. A. Saez-Nieto.** 2006. Prevalence and evolution of methicillin resistant *Staphylococcus aureus* in Spanish hospitals between 1996 and 2002. J. Clin. Microbiol. **44:**266–270.

57. **Vivoni, A. M., B. A. Diep, A. C. de Gouveia Magalhães, K. R. N. Santos, L. W. Riley, G. F. Sensabaugh, and B. Moreira.** 2006. Clonal composition of *Staphylococcus aureus* isolates at a Brazilian university hospital: identification of international circulating lineages. J. Clin. Microbiol. **44:**1686–1691.

58. **Voyich, J. M., K. R. Braughton, D. E. Sturdevant, A. R. Whitney, B. Saïd-Salim, S. F. Porcella, R. D. Long, D. W. Dorward, D. J. Gardner, B. N. Kreiswirth, J. M. Musser, and F. R. DeLeo.** 2005. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. J. Immunol. **175:**3907–3919.

59. **Waldron, D. E., and J. A. Lindsay.** 2006. SauI: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* of different lineages. J. Bacteriol. **188:**5578–5585.

60. **Zhang, K., J. A. McClure, E. Sameer, T. Louie, and J. M. Conly.** 2005. Novel multiple PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to IV in methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **43:**5026–5033.