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Comparison of Single- and Multilocus Sequence Typing and Toxin Gene Profiling for Characterization of Methicillin-Resistant *Staphylococcus aureus*▽

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We compared three novel methicillin-resistant *Staphylococcus aureus* (MRSA) genotyping methods with multilocus sequence typing (MLST) and *spa* typing to assess their utility for routine strain typing. The new methods were *femA* and *nuc* sequence typing and toxin gene profiling (TGP), using a multiplex-PCR-based reverse line blot assay to detect 13 pyrogenic superantigen and exfoliative toxin genes. Forty-two well-characterized MRSA strains, representing 15 MLSTs or 9 clonal clusters (CCs), were genotyped by all methods. Twenty-two *spa*, nine *femA*, and seven *nuc* sequence types were identified. The *femA* sequence types correlated exactly with CCs; *nuc* sequences types were less discriminatory but generally correlated well with *femA* types and CCs. Ten isolates contained none of 13 toxin genes; TGPs of the remainder comprised 1 to 5 toxin genes. The combination of *spa* typing and TGPs identified 26 genotypes among the 42 strains studied. A combination of two or three rapid, inexpensive genotyping methods could potentially provide rapid MRSA strain typing as well as useful information about clonal origin and virulence.

Methicillin-resistant *Staphylococcus aureus* (MRSA) genotyping is used to study its evolution and epidemiology and to assist in infection control (39). Different typing methods provide different information. Multilocus sequence typing (MLST) reveals slowly accumulating changes in conserved genes that reflect long-term evolutionary changes and can identify global spread of the relatively small number of successful clones (13). It has limited discriminatory power and is unsuitable for outbreak investigation, whereas pulsed-field gel electrophoresis is highly discriminatory and can identify recent changes. It is most widely used for outbreak investigation and infection control (9, 28). Both methods are relatively expensive and slow, and a number of rapid, inexpensive typing methods, based on sequence or length polymorphisms of variable genes or loci, have been described that are objective and relatively inexpensive. These include *coa* (41) and *spa* (38) sequence typing and the multilocus variable number tandem repeat assay (12, 29).

spa typing, which depends on differences in the number and sequence of tandem repeats in region X of the protein A gene (44), is discriminatory, rapid, inexpensive, and objective (25, 37, 41). The development of a shareable web-based database (www.spaServer.Ridom.de) (15) and the utility of *spa* typing for early-warning systems (31) have contributed to the rapid uptake of MRSA *spa* typing by diagnostic and public health laboratories.

In this study, we investigated the potential utility of two additional *S. aureus* gene polymorphisms for strain typing, namely, *femA*, one of several genes involved in the synthesis of the branched-peptide structure of *S. aureus* peptidoglycan (4), and *nuc*, which encodes an extracellular thermostable nuclease of *S. aureus* (5). Both are species-specific *S. aureus* genes; they have been widely used as PCR targets for identification (21), but their polymorphisms have not been widely investigated (14).

S. aureus produces numerous toxins, including enterotoxins or pyrogenic superantigens and exfoliative toxins, some of which are encoded by genes carried on staphylococcal pathogenicity islands and associated with certain clonal complexes (CCs), whereas genes encoding others, such as the Panton-Valentine leucocidin (PVL), are carried on bacteriophages and readily transferred between different lineages (26, 27). This suggests that a toxin gene profile (TGP) could help identify *S. aureus* CCs as well as providing information about virulence. Various molecular methods have been described for studying the distribution of staphylococcal toxins (2, 10).

We used 42 well-characterized MRSA strains to compare sequence polymorphisms of *femA* and *nuc* and TGPs, based on a multiplex PCR-based reverse line blot assay (mPCR/RLB) (22), with two established typing methods—namely, *spa* typing and MLST—to determine their potential utility for MRSA genotyping.

MATERIALS AND METHODS

***S. aureus* isolates.** We used 42 well-characterized reference and clinical *S. aureus* isolates in this study, as shown in Table 1, including 35 from various parts of Australia, provided by Philip Giffard, Cooperative Research Centre for Diagnostics, Queensland University of Technology, Brisbane, and Graeme Nimmo,

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TABLE 1. Genotypes and *spa* types of 42 well-characterized methicillin-resistant *S. aureus* isolates used in this study

Strain	GenBank accession no.[a]	Defined *spa* length[b]	*spa* type[c]	*spa* profile[c]	Clonal type[d,e]	Sources of Australian isolates[h]
B827549	EF094508	134	t1784	07-34-33-13	ST~1-SCC*mec*-new	QHPS
HU25[g]	EF094528	182	t138	08-16-02-25-17-24	ST239-SCC*mec*-IIIA	
HDG2[g]	EF094527	182	t421	15-12-16-02-25-17	ST239-SCC*mec*-IIIB	
K704540[f]	EF094525	206	t037	15-12-16-02-25-17-24	ST~239-SCC*mec*-III	QHPS
K711532[f]	= EF094525	206	t037	15-12-16-02-25-17-24	ST~239-SCC*mec*-III	QHPS
AH13[f]	= EF094525	206	t037	15-12-16-02-25-17-24	ST239-SCC*mec*-IIIA	AGAR
RDH81[f]	= EF094525	206	t037	15-12-16-02-25-17-24	ST239-SCC*mec*-IIIA	AGAR
AH1[f]	= EF094525	206	t037	15-12-16-02-25-17-24	ST128-SCC*mec*-IIIA	AGAR
RPAH 18[f]	= EF094525	206	t037	15-12-16-02-25-17-24	ST239-SCC*mec*-III	AGAR
RPAH15[f]	= EF094525	206	t037	15-12-16-02-25-17-24	ST239-SCC*mec*-III	AGAR
ANS46[g]	= EF094525	206	t037	15-12-16-02-25-17-24	ST239-SCC*mec*-III	
PC8[f]	EF094507	206	t127	07-23-21-16-34-33-13	ST1-SCC*mec*-IV	AGAR
FH43[f]	= EF094507	206	t127	07-23-21-16-34-33-13	ST~1-SCC*mec*-IV	AGAR
SJOG 30[f]	= EF094507	206	t127	07-23-21-16-34-33-13	ST1-SCC*mec*-IV	AGAR
RPH 85[f]	= EF094507	206	t127	07-23-21-16-34-33-13	ST~1-SCC*mec*-IV	AGAR
SN39[f]	= EF094507	206	t127	07-23-21-16-34-33-13	ST~1-SCC*mec*-new	AGAR
RHH58[f]	= EF094507	206	t127	07-23-21-16-34-33-13	ST~1-SCC*mec*-IV	AGAR
RHH10[f]	= EF094507	206	t127	07-23-21-16-34-33-13	ST~1-SCC*mec*-IV	AGAR
FH53[f]	= EF094507	206	t127	07-23-21-16-34-33-13	ST~1-SCC*mec*-I	AGAR
RPH2[f]	EF094510	206	t190	11-17-34-24-34-22-25	ST8-SCC*mec*-new	AGAR
PAH 58[f]	EF094514	230	t019	08-16-02-16-02-25-17-24	ST30-SCC*mec*-IV	AGAR
PAH 1[f]	= EF094514	230	t019	08-16-02-16-02-25-17-24	ST30-SCC*mec*-IV	AGAR
MW2[g]	EF094526	230	t128	07-23-23-21-16-34-33-13	ST1-SCC*mec*-IV	
RBH98[f]	EF094522	230	t202	11-17-23-17-17-16-16-25	ST93-SCC*mec*-IV	AGAR
13792-4492[f]	= EF094522	230	t202	11-17-23-17-17-16-16-25	ST~93-SCC*mec*-IV	QHPS
IP01M1081[f]	EF094523	230	t216	04-20-17-20-17-31-16-34	ST59-SCC*mec*-IV	QHPS
14176-5710[f]	EF094524	230	t1959[c]	15-21-12-16-02-25-17-16	ST~239-SCC*mec*-III	QHPS
B8-10[f]	EF094509	230	t711	04-21-17-34-24-34-22-25	ST~8-SCC*mec*-IV	QHPS
J710566[f]	EF094516	254	t065	09-02-16-34-13-17-34-16-34	ST45-SCC*mec*-V	QHPS
RPH 74[f]	EF094517	254	t123	09-02-16-34-13-16-34-16-34	ST45-SCC*mec*-V	AGAR
IP01M2046[f]	EF094519	254	t1958[c]	08-21-17-13-13-new-34-33-34	ST78-SCC*mec*-IV	QHPS
E804531[f]	EF094518	278	t002	26-23-17-34-17-20-17-12-17-16	ST5-SCC*mec*-IV	QHPS
CH97[f]	= EF094518	278	t002	26-23-17-34-17-20-17-12-17-16	ST73-SCC*mec*-IV	AGAR
BK2464[g]	= EF094518	278	t002	26-23-17-34-17-20-17-12-17-16	ST5-SCC*mec*-II	
IMVS 67[f]	EF094511	278	t008	11-19-12-21-17-34-24-34-22-25	ST8-SCC*mec*-V	AGAR
COL[g]	= EF094511	278	t008	11-19-12-21-17-34-24-34-22-25	ST250-SCC*mec*-I	
DEN2988[g]	= EF094511	278	t008	11-19-12-21-17-34-24-34-22-25	ST8-SCC*mec*-IVA	
F829549[f]	EF094521	278	t186	07-12-21-17-13-13-34-34-33-34	ST88-SCC*mec*-IV	QHPS
C801535[f]	EF094520	278	t325	07-12-21-17-34-13-34-34-33-34	ST88-SCC*mec*-new	QHPS
E822485[f]	EF094515	302	t018	15-12-16-02-16-02-25-17-24-24-24	ST36-SCC*mec*-II	QHPS
CH69[f]	EF094513	326	t1963[c]	26-23-13-17-31-29-17-25-17-25-16-28	ST~22-SCC*mec*-IV	AGAR
CH16[f]	EF094512	422	t032	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	ST22-SCC*mec*-IV	AGAR

[a] The relevant *spa* sequences have been submitted to GenBank; other strains with identical *spa* sequences are indicated by "=" and the accession no. for the corresponding submitted GenBank sequence.

[b] Defined *spa* lengths are the distances from the start and end points, equal to 1156 and 1481 in GenBank sequence J01786, which correlates with the start and end point of the suggested 5′ and 3′ signature sequences (www.spaServer.Ridom.de). The full repetitive region sequence length can be calculated by adding together the lengths of sequences of individual repeats.

[c] After comparison with *spa* database (www.spaServer.Ridom.de) and GenBank sequences, three new *spa* types sequences were identified and submitted to the *spa* database (www.spaServer.Ridom.de). Please refer to the *spa* database for *spa* type and profile nomenclature.

[d] ST, MLST; SCC*mec*, staphylococcal cassette chromosome *mec*. Information provided by strain donors; ST~, single nucleotide polymorphism type as described by Huygens et al. (18) using the computer program Minimum SNPs to compare with existing MLST data (17).

[e] Clonal type refers to the combination of ST and SCC*mec* type.

[f] Thirty-five Australian strains were provided by Philip Giffard, Cooperative Research Centre for Diagnostics, Queensland University of Technology, Brisbane, Australia, and Graeme Nimmo, Queensland Health Pathology Services, Princess Alexandra Hospital, Brisbane, and have been used in several previous studies (17, 18, 40).

[g] Seven isolates were provided by Herminia de Lencastre, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal, and have been used in several previous studies (33, 34); *spa* types identified in this study were identical with those previously reported for these strains (de Lencastre, personal communication).

[h] QHPS, Queensland Health Pathology Service (isolates from various diagnostic laboratories in Queensland); AGAR, Australian Group on Antibiotic Resistance (isolates from a study of community MRSA in Australia) (6).

Queensland Health Pathology Services, Princess Alexandra Hospital, Brisbane, Australia. Some have been used in several previous studies (40). Seven strains were provided by Herminia de Lencastre, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal, and also have been used in previous studies (33, 34) Two of these strains (MW2 and COL) have been fully sequenced (26). MLST and *SCCmec* typing results were provided by the donors of the strains (33, 34, 40).

DNA extraction. DNA extraction was performed as described previously (23).

Toxin gene detection. A well-established mPCR/RLB protocol developed in our laboratory (22) was used to detect 13 *Staphylococcus aureus* toxin genes.

TABLE 2. Primers and probes used in mPCR/RLB for detection of 13 toxin genes

Primer/probe[a]	Target	T_m °C[b]	GenBank accession no.	Primer/probe sequence (5′–3′)[c]	References[d]
nucSb	*nuc*	65.68	V01281	**511**GCG ATT GAT GGT GAT ACG GTT**531**	7
nucAp	*nuc*	61.36	V01281	**558**CAT TGG TTG ACC TTT GTA CAT TAA **535**	This study
nucSp	*nuc*	61.06	V01281	**745**GAT GGA AAA ATG GTA AAC GAA G**766**	This study
nucAb	*nuc*	69.12	V01281	**789**AGC CAA GCC TTG ACG AAC TAA AGC**766**	7
seaSb	*sea*	64.05	M18970	**487**CCT TTG GAA ACG GTT AAA ACG**507**	3
seaSp	*sea*	68.83	M18970	**531**GGA GTT GGA TCT TCA AGC AAG ACG **554**	3
seaAp	*sea*	63.87	M18970	**613**TCT GAA CCT TCC CAT CAA AAA C**592**	3
seaAb	*sea*	62.91	M18970	**691**TTGA ATA CTG TCC TTG AGC ACC**670**	43
sebSb	*seb*	64.82	M11118	**634**TCG CAT CAA ACT GAC AAA CG**653**	3
sebSp	*seb*	61.1	M11118	**662**GTAT GTA TGG TGG TGT AAC TGA GC**685**	43
sebAp	*seb*	60.06	M11118	**831**CA CCA AAT AGT GAC GAG TTA GG**810**	43
sebAb	*seb*	60.4	M11118	**924**CAT GTC ATA CCA AAA GCT ATT CTC**901**	3
secSb	*sec*	62.5	X05815	**664**G CTC AAG AAC TAG ACA TAA AAG CTA GG**690**	3
secSp	*sec*	63.13	X05815	**772**AAC GG(/a)C AAT ACT TTT TGG TAT GAT**795**	3
secAp	*sec*	61.4	X05815	**885**CTT CAC A(/t)CT TTT AGA ATC AAC CG**863**	43
secAb	*sec*	60.4	X05815	**935**TCA AAA TCG GAT TAA CAT TAT CC**913**	3
sedSb	*sed*	60.2	M28521	**332**CTA GTT TGG TAA TAT CTC CTT TAA ACG**358**	3
sedSp	*sed*	64.91	M28521	**360**TAA AGC CAA TGA AAA CAT TGA TTC A**384**	3
sedAp	*sed*	60.85	M28521	**491**CTT TTA TTT TCT CCT ATT ATT GG ATTTTT**463**	30
sedAb	*sed*	61.9	M28521	**653**CAA TTA ATG CTA TAT CTT ATA GGG TAA ACA TC**622**	3
seeSb	*see*	63.41	M21319	**424**C GAT TGA CCG AAG AAA AAA AAG**445**	30
seeSp	*see*	60.2	M21319	**479**CTA CAG TAC CTA TAG ATA AAG TTA AAA CAA GC**510**	3
seeAp	*see*	66.87	M21319	**613**TTT GCA CCT TAC CGC CAA AG**594**	3
seeAb	*see*	60.38	M21319	**659**TAA CTT ACC GTG GAC CCT TC**640**	3
segSb	*seg*	66.14	AF064773	**229**CAA CCC/T GAT CCT AAA TTA GAC GAA C**253**	2
segSp	*seg*	63.09	AF064773	**285**GGG AAC TAT GGG T(/a)AA TGT AAT GAA TC**310**	2
segAp	*seg*	62.61	AF064773	**338**CTT CCT TCA ACA GGT GGA GAC**318**	2
segAb	*seg*	62.91	AF064773	**485/401**GGA ACG CCA AAA ATG TCT ACT T**464/379**	35
sehSb	*seh*	60.86	U11702	**407**TTA GAA ATC AAG GTG ATA GTG GC **429**	2
sehSp	*seh*	61.25	U11702	**454**ACT GCT GAT TTA GCT CAG AAG TTT A **478**	2
sehAp	*seh*	60.1	U11702	**575**AGT GTT GTA CCT CCA TAT AGA C ATTC**550**	35
sehAb	*seh*	60.47	U11702	**641**TTT TGA ATA CCA TCT ACC CAA AC**619**	2
seiSb	*sei*	63.01	AY158703	**396**G GCC ACT TTA TCA GGA CAA TAC TT**419**	2
seiSp	*sei*	61.91	AY158703	**656**A CA C(a)TG GTA AAG GC(t)A AAG AAT ATG**679**	2
seiAp	*sei*	62.26	AY158703	**726**AAA ACT TAC AGG CAG TCC ATC TC**704**	2
seiAb	*sei*	58.23	AY158703	**818**AAT TAT CAT TAG TTA CTA TCT ACA TAT GAT ATT TC**784**	35
etaSb	*eta*	61.39	M17347	**374**CTA GTG CAT TTG TTA TTC AAG ACG**397**	3
etaSp	*eta*	69.51	M17347	**414**CCA TGC AAA AGC AGA AGT TTC AGC **437**	3
etaAp	*eta*	60.67	M17347	**492**TGC A(/g)TT GAC ACC ATA GTA CTT ATT C**468**	This study
etaAb	*eta*	62.72	M17347	**794**AAT GCT AAA TCA ACA CCT GC AC**773**	30
etbSb	*etb*	61.26	M17348	**190**TAC CAC CTA ATA CCC TAA TAA TCC AA**215**	3
etbSp	*etb*	61.37	M17348	**286**GAG ACA GTG CAT TAA ATG AAT AAC TTT**312**	3
etbAp	*etb*	62.41	M17348	**539**GAT TTC TTC TGC GCT GTA TTC TT**517**	This study
etbAb	*etb*	61.16	M17348	**609**C ATT ATC CGT AAT GTG TGT ATAAA GC**584**	43
etdSb	*etd*	61.75	AB057421	**5963**GCT CGG ATA CCC TTA TAA CTT TTC**5986**	This study
etdSp	*etd*	62.2	AB057421	**6055**CTG AGT CGG GAA ATT CTG G**6073**	43
etdAp	*etd*	61.47	AB057421	**6120**CAA CAT GAA TAC CA0A CTA ACT CTC C**6096**	This study
etdAb	*etd*	61.88	AB057421	**6259**CAT TAC TAA TGA GAC TGT AAT TCA GCT CT**6231**	This study
tsstSb	*tsst-1*	65.22	J02615	**348**AAG CCA ACA TAC TAG CGA AGG AAC**371**	3
tsstSp	*tsst-1*	60.5	J02615	**394**GGC GTT ACA AAT ACT GAA AAA TTA C**418**	30
tsstAp	*tsst-1*	64.36	J02615	**495**ATC GAA CTT TGG CCC(/a) ATA CTT T**474**	3
tsstAb	*tsst-1*	61.03	J02615	**556**GTA TTT GAG TTA GCT GAT GAC GAA**533**	43
pvlSb	*pvl*	65.29	X72700	**2651**TTT TAG GCT CAA GAC AAA GCA AC**2673**	This study
pvlAp	*pvl*	65.3	X72700	**2731**TAC CTC TGG ATA ACA CTG GCA TTT T**2707**	11
pvlSp	*pvl*	61.76	X72700	**2733**CTT CAA TCC AGA ATT TAT TGG TGT **2756**	11
pvlAb	*pvl*	65.8	X72700	**2783**TTT GCA GCG TTT TGT TTT CG**2764**	11

[a] S, sense; A, antisense; b, biotin labeled (all the primers were biotin labeled at the 5′ end); p, probe (all the probes were 5′ end C6 amine labeled).
[b] T_m values were provided by the primer synthesizer (Sigma-Aldrich).
[c] Boldface numbers represent the numbered base positions at which primer/probe sequences start and finish (starting at point "1" of the corresponding GenBank sequence). Underlined portions indicate modifications of published primer/probe sequences. The bases in parenthesis represent sequences with polymorphisms compared with GenBank sequences or our own sequencing results (for five probes with heterogeneous hybridization).
[d] Primers and probes were used as previously published (some with modification) except, as indicated, those designed for this study.

Target genes, primer and probe sequences, physical characteristics, and locations within selected GenBank sequences are shown in Table 2. All primers and probes had similar physical characteristics to allow simultaneous amplification and hybridization, respectively, in a multiplex reaction (22). Two gene-specific PCR primer pairs and two gene-specific probes were designed for each of 13 toxin genes. All primers were 5′ end biotinylated to allow detection of hybridization with a streptavidin peroxidase substrate. The probes were labeled with a 5′-end amine group to facilitate covalent linkage to the nylon membrane and to allow membranes to be stripped and reused repeatedly (22). Each multiplex reaction included *nuc* primers as the positive control for *S. aureus* and for quality control of DNA extraction and mPCR/RLB. All primers and probes were synthesized by Sigma-Aldrich (Sydney, Australia).

The mPCR/RLB was performed as previously described (22) with the following modifications: each 25-μl reaction mixture contained 0.5 U Hotstar *Taq* polymerase (QIAGEN, Melbourne, Australia), and the mPCR annealing temperature was optimized to 55°C.

Sequencing, sequence analysis, and phylogenetic tree. *femA*, *nuc*, and *spa* PCR primers were based on the published GenBank sequences using BioManager (http://biomanager.angis.org.au/). Sequencing was performed as described previously (24). For most targets, outer primers were used for amplification and inner primers for sequencing (Table 3).

The *spa* types were defined by reference to the shareable web-based database (www.spaServer.Ridom.de) (15). All *spa* repeat regions were submitted to the database, and *spa* types were assigned by the database by combining the sequences of all repeat regions.

TABLE 3. Primers used for PCR sequencing of *nuc*, *femA*, and *spa* genes

Primer	Target	T_m (°C)	GenBank accession no.	Primer sequence (5′–3′)[c]
nucS1[a]	*nuc*	60.3	V01281	**226**ATGACAGAATACTTATTAAGTGCTGG**251**
nucS2[b]	*nuc*	60.6	V01281	**232**GAATACTTATTAAGTGCTGGCATATG**257**
nucA1[b]	*nuc*	63.9	V01281	**908**TGACCTGAATCAGCGTTGTC**889**
nucA2[a]	*nuc*	63.7	V01281	**912**TTATTGACCTGAATCAGCGTTG**891**
femAS1[a]	*femA*	64.1	X17688	**577**ATGAAATTAATTAACGAGAGACAAATAGGAG**607**
femAS2[b]	*femA*	65.4	X17688	**591**CGAGAGACAAATAGGAGTAATGATAATGAAG**621**
femAA0[b]	*femA*	67.3	X17688	**1868**CTGTCTTTAACTTTTTTAAGTGCGGTATATGC**1837**
femAA[a]	*femA*	68.3	X17688	**1878**CTAAAAAATTCTGTCTTTAACTTTTTTAAGTGCGG**1844**
spaS[a]	*spa*	71.7	J01786	**1077**CTT CAT CCA AAG CCT TAA AGA CGA TCC TTC**1106**
spaA[a]	*spa*	71.4	J01786	**1543**CAA TTT TGTCAG CAG TAG TGC CGT TTG**1517**
spaSEQ[b]	*spa*	71.9	J01786	**1540**TTT TGTCAG CAG TAG TGC CGT TTG CT**1515**

[a] For most targets, outer primers were used for amplification and, less commonly, for sequencing.
[b] Inner primers were mainly used for sequencing, since they gave better results.
[c] Boldface numbers represent the numbered base positions at which primer sequences start and finish (starting at point "1" of the corresponding GenBank sequence).

Data obtained from different typing methods were recorded and stored in an Access file, which was imported into the BioNumerics software program (Applied Maths) with appropriate formatting. A phylogenetic tree was generated by using the categorical coefficient and clustered by the Ward algorithm.

Calculation of index of diversity. Simpson's index of diversity was calculated for each individual genotyping method and for combinations of methods, as described by Hunter and Gaston (16).

Nucleotide sequence accession numbers. The nearly full-length sequences (see below) of selected *femA* and *nuc* genes and partial *spa* sequences were deposited

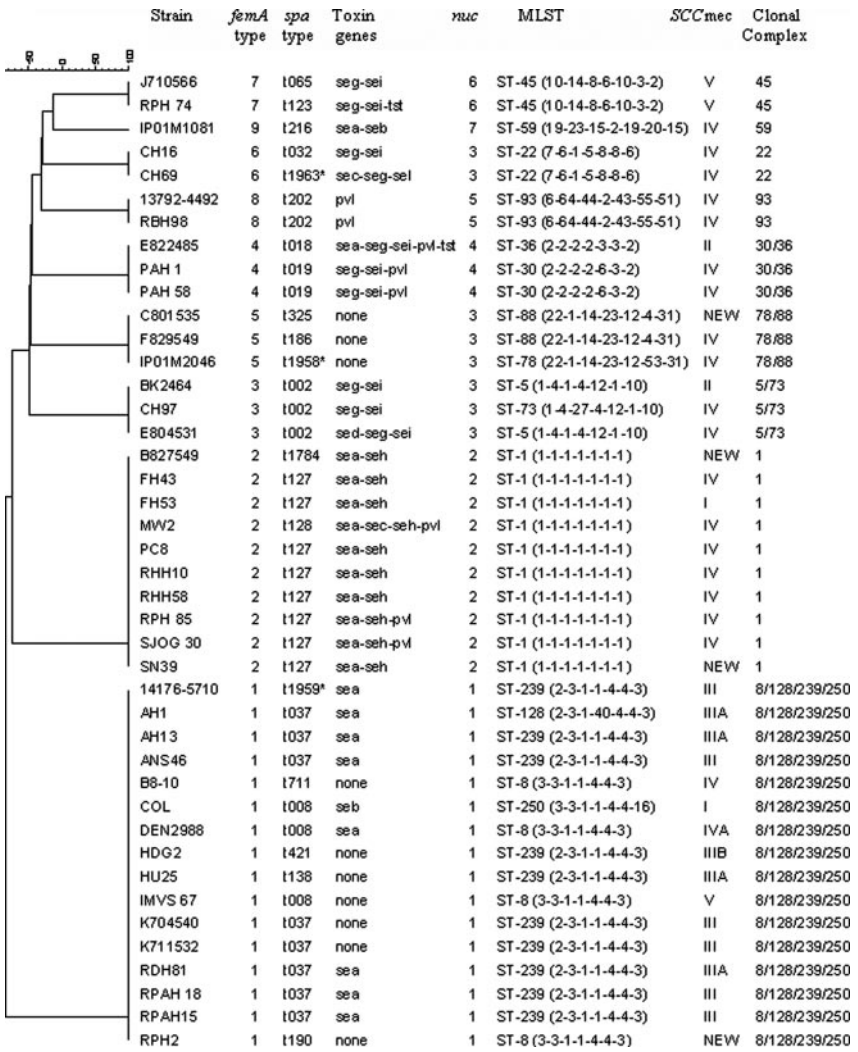

FIG. 1. Relatedness of 42 MRSA strains between different typing methods. *, strains CH 69, IPO1M2046, and 14176-5710 belonged to *spa* types t1963, t1958, and t1959, respectively, which have not been previously deposited in the database.

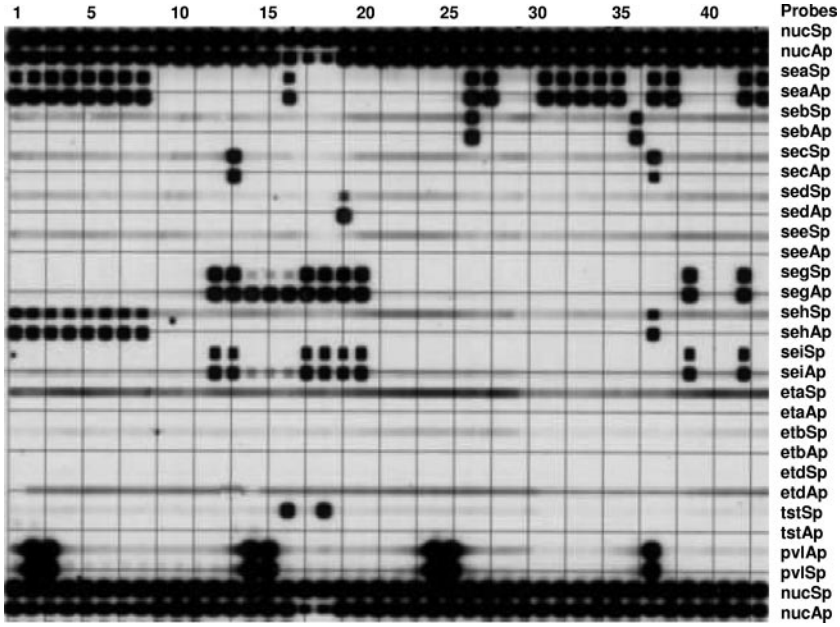

FIG. 2. The 13 toxin gene profiles of the 41 strains. Lanes 1 to 42 show results for the following isolates, in order (see Table 1): FH43, SJOG 30, RPH85, B827549, SN39, RHH58, RHH10, FH53, B8-10, RPH2, IMVS67, CH16, CH69, PAH58, PAH1, E822485, J710566, RPH74, E804531, CH97, IP01M2046, C801535, F829549, RBH98, 13792-4492, IP01M1081, 14176-5710, K704540, K711532, AH13, RDH81, AH1, RPAH18, RPAH15, COL, MW2, DEN2988, BK2464, HDG2, HU25, a control strain, and ANS46.

in GenBank with the following accession numbers: for *femA*, DQ103589 and DQ352456 to DQ352463; for *nuc*, DQ507377 to DQ507382; and for *spa*, EF094507 to EF094528. Eight *S. aureus* genome sequences were used for reference: AJ938182 (RF122), NC_002952 (MRSA252), AF144661 (*Staphylococcus aureus* subsp. *anaerobius*), NC_002745(N315), NC_002758 (Mu50), NC_003923 (MW2), NC_002951 (COL), and NC_002953 (MSSA476).

RESULTS AND DISCUSSION

Sequence polymorphisms of *femA* and *nuc*. Nine *femA* sequence types with lengths of approximately 1,215 bp were identified, of which five were newly identified and four had been previously deposited in GenBank. A total of 39 polymorphism sites were found among those sequences. Seven *nuc* sequence types of approximately 700 bp were identified, with 28 polymorphisms sites. Four sequence types had not been previously identified.

***spa* types and TGPs.** Twenty-two *spa* types were identified, and sequences of each new type were submitted to GenBank with accession numbers shown in Table 1. Nineteen of the *spa* types were already recorded in the *spa* database (www.spaServer.Ridom.de) (15), and three were new types, first identified in Australian strains (Table 1; Fig. 1). There were 14 different TGPs (Fig. 1 and 2).

Comparison of MLST, *femA* and *nuc* sequence types, *spa* types, and TGPs. MLST are based on sequences of seven housekeeping genes (http://www.mlst.net/). Isolates with identical sequences for all seven genes are considered to be clonal and those with five or six matching genes to belong to the same CC (27). There were 15 MLST and nine CCs among the 42 strains studied (Table 1). The latter correlated exactly with *fem*A sequence types, suggesting that *fem*A sequencing may be a useful "shorthand" single-locus surrogate for MLST (Fig. 1). In future, informative single nucleotide polymorphisms in the *femA* sequence may be able to predict *femA* type and CC. One of the candidate methods is rolling-circle amplification, which has been used successfully in our laboratory (42).

There were seven *nuc* sequence types, which were therefore less informative. One sequence type was represented among three *femA* sequence types (and corresponding CCs).

The relationships between MLST and TGP varied (Fig. 1). No toxin genes were found in 10 isolates belonging to four sequence types (STs) (ST-8, -78, -88, and -239). One to five toxin genes were found in various combinations in the remaining isolates. Some STs included more than one TGP, e.g., ST-5, -22, -45, and -239 each included isolates with two different TGPs, which reflects the ability of mobile genetic elements on which toxin genes are carried to transfer laterally between clones.

However, some toxin genes are transferred vertically within specific CCs (32, 36). For example, all 10 ST-1 isolates (but none belonging to other STs) contained *sea* and *seh*; *sea* alone was found in another eight isolates belonging to CC 8/239. *seg* and *sei*, which are part of the enterotoxin gene complex (*egc*), were always present together, in 10 isolates spread among four CCs (ST-5/73, -22, -45, and -30/36). This is consistent with a previous report that *egc* is preferentially distributed among CCs 5 and 30/36 (19). In addition, we identified mutations in regions of *seg* and *sei* probes in isolates belonging to CC 30/36.

The Panton-Valentine leukocidin gene (*pvl*) was identified, with a variety of other toxin genes (depending on ST/CC), in eight isolates belonging to ST-1 (three isolates), ST-93 (two isolates), or CC 30/36 (three isolates); seven of eight PVL-containing isolates belonged to SCC*mec* type IV, which is generally associated with community-acquired MRSA. PVL is associated with necrotic skin and soft tissue lesions and, more

TABLE 4. Comparison of discriminatory powers of each genotyping method and various combinations of methods for 42 MRSA strains using Simpson index of diversity

Genotyping method(s)	No. of types	% of largest type	DI[a]
Individual			
SCC*mec*	9	45.2	0.764
nuc sequence types	7	38.1	0.77
femA sequence types	9	38.1	0.794
TGPs	14	23.8	0.88
MLST	15	23.8	0.882
spa types	22	19	0.926
Combinations			
femA-spa-TGP	27	14.3	0.959
TGP-*spa*	27	14.3	0.959
TGP-*spa-nuc*	27	14.3	0.959
TGP-*spa*-SCC*mec*	30	9.5	0.98
femA-spa-TGP-SCC*mec*	31	9.5	0.981
femA-spa-TGP-*nuc*-MLST-SCC*mec*	34	9.5	0.987

[a] DI, Simpson index, calculated according to the method of Hunter and Gaston (16).

recently, with life-threatening necrotizing pneumonia and sepsis due to community-acquired MRSA (8, 44).

There were 22 *spa* types among the 42 strains tested. When combined with TGP, some *spa* types were further subdivided, making a total of 26 genotypes. For example, isolates belonging to *spa* type t002 contained two TGPs (*seg-sei* and *sed-seg-sei*), and those belonging to t008 contained three (*sea*, *seb*, and none). The combination of these two methods thus provides a high level of discrimination, using relatively inexpensive, rapid methods.

Comparison of discriminatory powers of each genotyping method and various combinations (Table 4) showed that *spa* typing is the most discriminatory. The addition of TGPs alone or TGP plus SCC*mec* typing increases the discriminatory power, but there is little additional increase from additional *femA* or *nuc* sequence typing.

Significance of sequence polymorphisms. These results indicate that *femA* and to some extent *nuc* sequence types correlate closely with MLST in this set of MRSA isolates, suggesting that these genes evolve at a rate similar to that of housekeeping genes within CCs (20, 27). *spa* types were more discriminatory for strain typing but correlated less well with MLST or CCs.

Significant sequence variation in *femA* and *nuc* also has potential implications for their use as species-specific PCR targets for identification of *S. aureus.* The possibility of mutations needs to be considered in the design of probes and primers to avoid false-negative or inaccurate quantitative PCR results. Our results show that some mutations occurred in the region of primers used as species-specific primers (1).

There were significant sequence polymorphisms in *femA* and *nuc* genes, which have not been previously well studied, which has potential implications for their use as species-specific PCR targets for identification of *S. aureus*. Both correlated well with each other, and *femA* sequence types correlated with MLST/CCs. TGPs provide useful information about potential virulence and the evolutionary history of *S. aureus* strains and can increase the discriminatory power of *femA* and *spa* sequence typing. Prospective testing of unselected clinical isolates will be needed to adequately determine the optimal combination of methods for MRSA surveillance.

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