JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2009, p. 577–585 Vol. 47, No. 3
0095-1137/09/$08.00+0 doi:10.1128/JCM.01347-08
Copyright © 2009, American Society for Microbiology. All Rights Reserved.

Multiplexed Genotyping of Methicillin-Resistant *Staphylococcus aureus* Isolates by Use of Padlock Probes and Tag Microarrays[▽]

Kevin Kurt,[1] Anders Alderborn,[2] Mats Nilsson,[2] Birgit Strommenger,[1] Wolfgang Witte,[1] and Ulrich Nübel[1]*

Robert Koch Institute, Wernigerode, Germany,[1] and Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden[2]

Received 15 July 2008/Returned for modification 30 July 2008/Accepted 13 January 2009

We developed and tested a ligase-based assay for simultaneous probing of core genome diversity and typing of methicillin resistance determinants in *Staphylococcus aureus* isolates. This assay uses oligonucleotide padlock probes whose two ends are joined through ligation when they hybridize to matching target DNA. Circularized probes are subsequently amplified by PCR with common primers and analyzed by using a microarray equipped with universal tag probes. Our set of padlock probes includes oligonucleotides targeting diagnostic regions in the *mecA*, *ccrB*, and *ccrC* genes of the SCC*mec* cassette in methicillin-resistant *S. aureus* (MRSA). These probes determine the presence and type of SCC*mec* cassettes (i.e., SCC*mec* types I to VI). Additional oligonucleotides interrogate a number of highly informative single nucleotide polymorphisms retrieved from a multilocus sequence typing (MLST) database. These latter probes enable the exploration of isolates' phylogenetic affiliation with clonal lineages of MRSA as revealed by MLST. The described assay enables multiplexed genotyping of MRSA based on a single-tube reaction. With a set of clinical isolates of MRSA and methicillin-susceptible *S. aureus* ($n = 66$), 100% typeability and 100% accuracy were achieved. The assay described here provides valuable genotypic information that may usefully complement existing genotyping procedures. Moreover, the assay is easily extendable by incorporating additional padlock probes and will be valuable for the quick and cost-effective probing of large numbers of polymorphisms at different genomic locations, such as those ascertained through currently ongoing mutation discovery and genome resequencing projects.

Methicillin-resistant *Staphylococcus aureus* (MRSA) causes significant morbidity and mortality. Genotyping of MRSA isolates is necessary to detect outbreaks and to monitor epidemiological trends. Various methods based on DNA sequencing have more recently been applied for typing, sharing the advantages of excellent interlaboratory reproducibility and data portability (1, 7, 8, 20, 39).

Multilocus sequence typing (MLST) is based on sequence analyses of seven metabolic housekeeping genes. Strains with identical sequences at all seven genetic loci are assigned unique sequence types (8). To date, MLST has been applied to several thousand isolates of *S. aureus*. Accumulated data indicate that the vast majority of MRSA strains are affiliated with a small number of clusters of closely related sequence types that are referred to as "clonal complexes" (9). Clones of MRSA have been defined based on the combination of MLST with PCR-based analyses of the genetic element causing methicillin resistance, SCC*mec* (staphylococcal chromosome cassette) (7, 32). Classification of SCC*mec* diversity is based on variation in the *ccr* gene complex, which includes recombinase genes that are required for SCC*mec* mobility, and the *mec* gene complex, which includes the *mecA* gene that causes methicillin resistance (6, 17, 18). Since recombinase gene sequences are conserved within SCC*mec* types, analysis of *ccrB* and *ccrC* genes enables sequence-based typing of SCC*mec*, concordant with PCR-based classification (21, 28, 29).

The limited discriminatory power of MLST and the high costs associated with sequence analyses of seven slowly evolving genetic loci have driven the development of a number of sequence-based typing approaches that rely on the investigation of single highly variable genomic regions (5, 10, 19, 35). Among these, the most frequently applied method explores a polymorphic repetitive region in the staphylococcal protein A (*spa*) gene (1, 7, 40). While currently being widely applied for investigations of both local outbreaks and global epidemiology of MRSA, *spa* typing in several cases has been demonstrated to provide misleading results due to recombination events that have caused the horizontal transfer of *spa* genes between unrelated strains (11, 31, 39). In addition, in hypervariable DNA sequences such as the tandem repeat region of the *spa* gene, homoplasies frequently emerge (23). These limitations of single-locus-based procedures result in the requirement for analyses of additional loci if typing reliability is to be improved (7, 11, 39).

Giffard and coworkers recently retrieved highly informative sets of single nucleotide polymorphisms (SNPs) from MLST databases (16, 30, 38). Through determination of the status of a very limited number of these SNPs by applying real-time PCR, they could classify MRSA strains into clusters consistent with the population structure of *S. aureus*, including all major clonal complexes and prevalent subclusters (16, 38). Analysis of these stable phylogenetic markers may be a useful amendment to typing procedures relying on more variable genomic

* Corresponding author. Mailing address: Robert-Koch-Institut, Burgstr. 37, 38855 Wernigerode, Germany. Phone: 03943-679338. Fax: 03943-679317. E-mail: nuebelu@rki.de.
▽ Published ahead of print on 21 January 2009.

regions such as the *spa* gene. While retaining important advantages of sequencing-based methods including data portability and ease of interpretation, the determination of these SNPs can be achieved more cost-effectively and rapidly than DNA sequencing and is more readily amenable to high throughput.

Many methods of SNP determination exist, most of which rely on locus-specific PCR amplification and hence are inherently difficult to perform in a multiplexed format (41). In contrast, oligonucleotides that circularize in sequence-specific ligation reactions (padlock probes) have been used to analyze SNPs in genomic DNA without prior target amplification (4, 22). Subsequent to ligation, circularized padlock probes may be PCR amplified with universal primers and the products may be identified by use of microarrays (4). The combination of multiple, allele-specific padlock probes in single reactions has been demonstrated to enable the determination of more than 1,000 SNPs in parallel (12).

In the present study, we have analyzed informative SNPs retrieved from MLST data by applying a padlock probe assay adopted from human genomics (4). In addition, we have included probes for diagnostic regions of recombinase genes (*ccrB* and *ccrC*) from SCC*mec* elements within the same assay. The resulting assay enabled the multiplexed simultaneous determination of an isolate's phylogenetic affiliation, consistent with MLST, and its complement and type of methicillin resistance determinant, concurrent with PCR-based classification of SCC*mec* elements.

TABLE 1. Bacterial isolates used in this study

Isolate	MLST	Lineage	SCC*mec*	*spa*
03-02773	1	CC 1	IV	t175
06-00468	5	CC 5	VI	t311
06-01359	5	CC 5	VI	t640
03-02106	5	CC 5	V	t002
02-01587	5	CC 5	II	ND[a]
97S101[b]	5	CC 5	III	t045
HDE 288	5	CC 5	VI	t311
Mu50	5	CC 5	II	t002
N315	5	CC 5	II	t002
05-02212	225	ST 225	II	t003
04-02981	225	ST 225	II	t003
05-02010	710	ST 225	II	t003
05-01977	228	ST 228	I	t001
04-03631	228	ST 228	I	t001
98-00131	228	ST 228	I	t001
98-11552	228	ST 228	I	t001
05-02040	8	CC 8	IV	t008
NCTC 8325	8	CC 8	MSSA	t211
93/1000[b]	254	CC 8	IV	t009
93-00635	239	CC 239	III	t037
NCTC 11939	239	CC 239	III	t037
94-01450	247	CC 8	I	t051
93-00134	247	CC 8	I	t051
04-00608	22	CC 22	IV	t310
03-00397	22	CC 22	IV	t032
90/10685[b]	22	CC 22	IV	t022
03-01621	30	CC 30	MSSA	t021
MSau192	30	CC 30	MSSA	t274
99-159[b]	36	CC 30	II	t018
C2Sau77	605	CC 30	MSSA	ND
02-02756	45	CC 45	IV	t038
02-02712	47	CC 45	IV	t026
05-02065	59	CC 59	MSSA	t1151
06-01240	375	CC 59	V	t172
97-00825	80	CC 80	IV	t044
00-01488	51	CC 121	MSSA	t269
00-01004	121	CC 121	MSSA	t159
95-00544	427	CC 121	MSSA	t159

[a] ND, not determined.
[b] Isolate from the Harmony collection.

MATERIALS AND METHODS

Bacterial isolates, DNA extraction, and genotyping procedures. Staphylococcal isolates were selected from the collection at the German reference center for staphylococci and from the international Harmony collection (Tables 1 and 2). They are representative of clonal lineages that are most prevalent in Germany and central Europe. Lysostaphin (100 mg/liter) was applied to achieve lysis of bacterial cells from overnight cultures, and DNA was extracted by using the DNeasy tissue kit (Qiagen). DNA concentration was determined photometrically. Multilocus sequence typing was performed as described previously (8). Affiliation with clonal complexes was determined by using the software eBURST (9). SCC*mec* element types I to V were identified by applying a combination of PCRs targeting diagnostic regions (43, 45). Briefly, the *ccr* type and class of the *mec* gene complex were identified as described by Okuma et al. (26) and Ito et al. (18) and additional features were determined for confirmation (14, 27). SCC*mec* type VI was identified by sequencing the *ccrB* gene as recently described (28).

Oligonucleotides. Padlock probes were designed on the basis of sequences available from the MLST database (www.mlst.net) or the GenBank database (www.ncbi.nlm.nih.gov), respectively, by using the Probemaker software (36) (kindly provided by Johan Stenberg). Probemaker is particularly useful for designing padlock probes because it provides for the incorporation of multiple so-called "tag sequences," sequence elements that function as target regions for, e.g., allele-specific primers and locus-specific microarray hybridization (Fig. 1) (36). The padlock probe sequences used are listed in Table 3. For 5′ phosphorylation of padlock probes, an equimolar mixture of oligonucleotides (10 μM) was incubated with T4 polynucleotide kinase (0.2 U/μl), kinase reaction buffer A, and 1 mM dATP for 45 min at 37°C according to the manufacturer's recommendations (Fermentas). PCR primers with the following sequences were used: universal primer Pr-F, TAC TGA GGT CGG TAC ACT CT; allele 1 primer Pr-R1, TCA GTA GCC GTG ACT ATC GA; allele 2 primer Pr-R2, TCA GTA CAT GAA TCC GTA GT. Primers Pr-R1 and Pr-R2 were labeled at their 5′ ends with the fluorescent cyanine dyes Cy5 and Cy3, respectively. For microarray hybridization control, a previously described oligonucleotide, RC-Bwm-111, with the sequence ATA AAT TCC GCA ATT TGT ATG and labeled with cyanine Cy3 was used (24). It was reverse complementary to the hybridization control probe Bwm-111 (CAT ACA AAT TGC GGA ATT TAT) affixed to the microarray. Tag oligonucleotide sequences were selected from the GeneFlex Tag Array collection (Affymetrix). Fluorescently labeled oligonucleotides were purchased from Metabion (Munich, Germany). All other oligonucleotides were synthesized and purified by high-pressure liquid chromatography (Invitrogen, Karlsruhe, Germany).

Ligation, exonucleation, and amplification. The principles and reaction steps of the padlock probe assay are illustrated in Fig. 1. This assay was modified from the protocol provided by Banér et al. (4). For ligation, bacterial DNA (5 ng/μl) was incubated with the mixture of padlock probes (300 pM each) and thermostable Ampligase (0.4 U/μl; Epicentre) in Ampligase reaction buffer supplied with 80 mM KCl at 95°C for 5 min, followed by 50°C for 60 min and 95°C for 2 min. For removal of nonligated probes, exonuclease I (final concentration, 0.125 U/μl; Fermentas), exonuclease I buffer, and bovine serum albumin (50 ng/μl) were added to the reaction mixture and the solution was incubated at 37°C for 60 min and, to inactivate the exonuclease, at 95°C for 10 min. For PCR amplification, 5 μl of this reaction mixture was added to 20 μl of a PCR mixture containing 0.0625 U/μl Hot-*Taq* DNA polymerase (Peqlab, Erlangen, Germany), Hot-*Taq* reaction buffer, 2.5 mM $MgCl_2$, 0.25 mM deoxynucleoside triphosphates, and 1.25 μM each of primers Pr-F, Pr-R1, and Pr-R2. Temperature cycling included 3 min at 96°C; 30 cycles of 30 s at 96°C, 30 s at 55°C, and 30 s at 72°C; and a final elongation for 2 min at 72°C.

Microarray fabrication, hybridization, and analysis. The hybridization control probe Bwm-111 (24) and tag oligonucleotide probes were resuspended in spotting buffer [12.5% dimethyl sulfoxide, 15 mM methylimidazole, 2.5 mg/ml *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide] and delivered onto aminosilane-coated glass slides (Nexterion A+; Schott, Germany) at eight replicate positions as described previously (2), with a TopSpot E microarrayer (Biofluidix, Germany). Microarray hybridization and washing, scanning with an Axon 4100B

TABLE 2. Bacterial isolates used as blinded samples

Isolate	MLST	Lineage	SCC*mec*	*spa*
05-01290	1	CC 1	MSSA	t127
06-01198	1	CC 1	MSSA	t321
06-01829	1	CC 1	MSSA	t1491
61974-Finland[a]	5	CC 5	II	t002
97120[a]	8	CC 8	IV	t008
97151[a]	8	CC 8	IV	t024
97392[a]	8	CC 8	IV	t008
98-01442	254	CC 8	IV	t009
3680[a]	239	CC 239	III	t037
3717[a]	239	CC 239	III	t037
04-02080	239	CC 239	III	t037
06-01537-1	241	CC 239	III	t363
97118[a]	247	CC 8	I	t051
134/93[a]	247	CC 8	I	t051
97S96[a]	247	CC 8	I	t054
98/26821[a]	22	CC 22	IV	t1275
04-03100	22	CC 22	IV	t032
90/10685[a]	22	CC 22	IV	t022
98/10618[a]	22	CC 22	IV	t022
98/24344[a]	22	CC 22	IV	t032
99/1139[a]	36	CC 30	II	t018
06-02026	34	CC 30	MSSA	t136
96/32010[a]	36	CC 30	II	t018
99/159[a]	36	CC 30	II	t018
AO 17934/97[a]	30	CC 30	IV	t021
06-01057	617	ST 617	IV	t305
825/96[a]	45	CC 45	IV	t004
02-02404	80	CC 80	IV	t044

[a] Isolate from the Harmony collection.

scanner, and data acquisition and processing by using the scanner's operating software GenepixPro 6.0 (Axon Instruments) were performed as described previously (2). To provide for a positive hybridization control, oligonucleotide RC-Bwm-111 was added to the hybridization mixture at a concentration of 5 fmol/μl; this oligonucleotide was complementary to the hybridization control probe Bwm-111 on the microarray and fluorescently labeled (24). Control of the ligation-and-amplification process was enabled through the inclusion of a pair of padlock probes directed against the staphylococcal 16S rRNA gene.

The medians of local background-corrected feature intensities (net intensities) and their signal-to-noise ratios (net intensities/standard deviation of the local background) were the basis of further calculations. Signal-to-noise ratios larger than 3 were required at the hybridization control probe, at the tag probe targeting the 16S rRNA gene, and at all tag probes targeting SNPs. Ratios of median net intensities measured at wavelengths of 635 and 532 nm were normalized to the ratio at probes for the staphylococcal 16S rRNA gene. The normalized ratios of median net intensities were averaged from eight replicate spots from the same microarray slide. SNP character state determination was based on clustering of data points in scatterplots (Fig. 2).

The probe for *mecA* met the requirement for positive detection if it displayed a signal-to-noise ratio higher than 3 (2). Recombinase genes *ccrB* and *ccrC* additionally were required to display the strongest fluorescence signal among all of the probes targeting recombinase genes (see Results and Discussion). Accordingly, *ccrB* gene probes with minor signals were considered negative. For *ccrC*, it was factored in that this gene may occur in isolation in SCC*mec* type V or in combination with *ccrB* from other SCC*mec* types (6).

Computer-aided evaluation of SNP subsets. To evaluate the theoretical performance of subsets of SNPs, the software package Minimum SNPs (30) was applied to concatenated sequences from MLST loci, which had been downloaded from the *S. aureus* MLST database at www.mlst.net. Minimum SNPs software was designed to identify the most informative polymorphisms within a given database, which may be useful for optimizing genotyping protocols (30). We used the implemented software modules "D method" (which calculates the extent of diversity that is discriminated on the basis of a given subset of SNPs) and the "backwards method" (which searches and lists all sequence types matching an input SNP profile) to determine the discriminatory power and specificity of SNP subsets, respectively.

RESULTS AND DISCUSSION

Probe and assay design. The developed assay included padlock probes for highly informative SNPs within MLST loci, probes for diagnostic regions of the SCC recombinase genes *ccrB* and *ccrC*, a probe for the gene *mecA*, which encodes penicillin-binding protein 2′, and probes for staphylococcal ribosomal DNA (Table 2). The lengths of the padlock probes used varied from 84 to 92 nucleotides. Each probe contained target-complementary segments at both ends, a locus-specific tag sequence complementary to one of the tag oligonucleotides affixed to the microarray, and two sequence elements targeted by PCR primers (Fig. 1 and Table 2). Target regions for forward and reverse primers overlapped by six nucleotides to reduce the total padlock probe length.

The assay's reaction steps are depicted in Fig. 1. A mixture

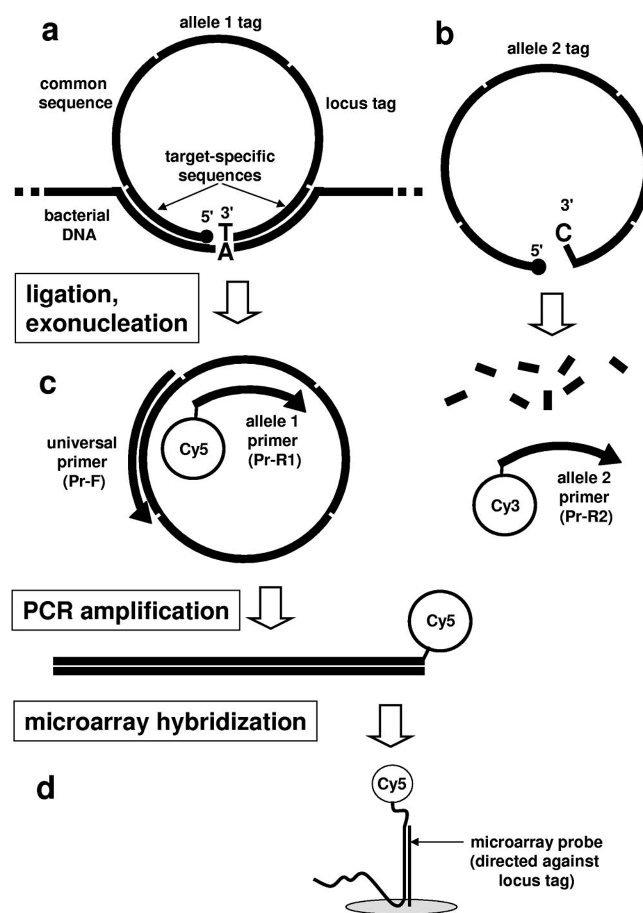

FIG. 1. Schematic depiction of the reaction steps involved in the padlock probe assay. This example shows a pair of probes that discriminate the two alleles at an adenosine/guanosine polymorphism. (a) When a padlock probe hybridizes to complementary target DNA, the probe's two ends are brought into juxtaposition. Ligation of adjacent ends produces a circular DNA molecule. (b) In contrast, padlock probes that do not match the target DNA are not circularized and hence are removed through subsequent exonucleation. (c) Circular padlock probes can be PCR amplified by using one universal primer and one of two allele-specific primers that are labeled with different fluorescent dyes. (d) Amplification products are identified through hybridization to probes on a microarray that are complementary to the padlock probes' locus tags.

TABLE 3. Sequences of the padlock probes used in this study

Probe[a]	5′ end	Common sequence[b]	Allele tag[c]	Locus tag	3′ end
Probes targeting SNPs at MLST loci					
tpi-243-A	GCATCTTCAGATGTTGAT	C1	A2	ATTTGATCGTAACTCGGGTG	AAATGCACACATTTCATTT
tpi-243-G	GCATCTTCAGATGTTGAT	C1	A1	ATTTGATCGTAACTCGGGTG	AAATGCACACATTTCATTC
yqiL-333-C	AAGCGACTGTTGTTGA	C1	A1	TTTAGTCGTTTGCCCGAGGC	GTCCCATTTTAAAACCG
yqiL-333-T	AAGCGACTGTTGTTGA	C1	A2	TTTAGTCGTTTGCCCGAGGC	TGTCCCATTTTAAAACCA
gmk-129-T	ATTAAAGCTTCAAACGCA	C1	A2	CTTAACTATTAGCGTCGGTG	ATTCTATAAATTGGTCATCTTTA
gmk-129-C	ATTAAAGCTTCAAACGCA	C1	A1	CTTAACTATTAGCGTCGGTG	TCTATAAATTGGTCATCTTTG
arcC-162-T	ACGATTGTGCCTACAG	C1	A1	CTATATCCTTACCGCGTATG	CTACTTCCACACGTGTA
arcC-162-A	ACGATTGTGCCTACAG	C1	A2	CTATATCCTTACCGCGTATG	CTACTTCCACACGTGTT
arcC-210-T	GTTGGGTTATTGAATCGT	C1	A2	GAGTAGCCTTCCCGAGCATT	AAAAGGACCAATTGGTTTA
arcC-210-C	GTTGGGTTATTGAATCGT	C1	A1	GAGTAGCCTTCCCGAGCATT	AAAGGACCAATTGGTTTG
aroE-132-A	TCTTTATGAGGAATTGTGAT	C1	A2	AACAACGATGAGACCGGGCT	TAAATACGGTATGATACGT
aroE-132-G	TCTTTATGAGGAATTGTGAT	C1	A1	AACAACGATGAGACCGGGCT	AAATACGGTATGATACGC
gmk-318-T	TCTAAACTTGGAGGTGC	C1	A2	AATACGCTGAATAGAGCCCT	TCGCTCTCTCAAGTGA
gmk-318-A	TCTAAACTTGGAGGTGC	C1	A1	AATACGCTGAATAGAGCCCT	TCGCTCTCTCAAGTGT
pta-294-C	CCTGTTGAATGTGCTG	C1	A1	ACATATCCTAATACAGGCGC	GGACGCACAGTGTCG
pta-294-A	CCTGTTGAATGTGCTG	C1	A2	ACATATCCTAATACAGGCGC	GGACGCACAGTGTCT
tpi-36-T	TGCGCTTTTTTGTTAATTT	C1	A2	GACGATCCTTATACTCGATG	CCATGTTTGAAAATAGCA
tpi-36-C	TGCGCTTTTTTGTTAATTT	C1	A1	GACGATCCTTATACTCGATG	CATGTTTGAAAATAGCG
pta-383-T	CATCACCTTTAATCATAAAG	C1	A2	GAAGACAGTTATACCCATGC	TCACCAAAGATGTATTGTA
pta-383-A	CATCACCTTTAATCATAAAG	C1	A1	GAAGACAGTTATACCCATGC	CACCAAAGATGTATTGTT
tpi-153-C	CCTGCAACAGCTTTCT	C1	A1	TGTCTACCTTTCCGTCAAGA	TTTAAGTTGATCTTCAGATAAG
tpi-153-T	CCTGCAACAGCTTTCT	C1	A2	TGTCTACCTTTCCGTCAAGA	TTTAAGTTGATCTTCAGATAAA
tpi-087-A	TCTGTTTCACCAACACA	C1	A1	AAACCATCGACTCACGGGAT	CACTTTCACGCTCTTCT
tpi-087-C	TCTGTTTCACCAACACA	C1	A2	AAACCATCGACTCACGGGAT	ACTTTCACGCTCTTCG
yqiL-505-T	TACTGCTTTATGTTGTGAG	C1	A1	ATTGACCAAACTGCGGTGCG	CATTTTGCTGTGCACA
yqiL-505-C	TACTGCTTTATGTTGTGAG	C1	A2	ATTGACCAAACTGCGGTGCG	CATTTTGCTGTGCACG
Probes targeting genes within SCC*mec* (recombinase genes and *mecA*)					
ccrB-I	AATTTTTGACTTCCATACG	C1	A1	ATAAAGCTCTATACTCCGCG	GCATTAACTTGCCTGTTG
ccrB-III	TTGTATTGACTTCCATACG	C1	A2	ATAAAGCTCTATACTCCGCG	GCATCAATTTACCACTGC
ccrB-II	TCATGTTTACTATTAGGTATTT	C1	A1	AACTAGCATCTAAGCACTCG	CATGTTGATTAATCATCAGT
ccrB-IV	TCGTGTTTACTATCTGGTA	C1	A2	AACTAGCATCTAAGCACTCG	CATGTTGGTTAATCATTAGC
ccrC-V	ACTTTTACCACTTCTTCG	C1	A1	GCTCTAATGTTAAGTGCTCG	CCAATATCACATCATTTTTATC
ccrB-VI	TACTTTATCATAGCCTAGAG	C1	A2	GCTCTAATGTTAAGTGCTCG	AGCTCTTTTTTACTATCAGG
mecA	ATGAAGGTGTGCTTACA	C1	A2	ACCACAGATCGAAGGTCGGA	CATAAATGGATAGACGTCAT
Probes targeting staphylococcal 16S rRNA gene					
rDNA-Cy5	CGTAGGAGTCTGGACC	C1	A1	AAGGCACGTATCATATCCCT	CCTACTGCTGCCTCC
rDNA-Cy3	CGTAGGAGTCTGGACC	C1	A2	AAGGCACGTATCATATCCCT	CCTACTGCTGCCTCC

[a] Probe names are composed of gene names, SNP positions within genes, and alleles.
[b] Common sequence C1, AGAGTGTACCGACCTCAGTA.
[c] Allele tag A1, GCCGTGACTATCGA; allele tag A2, CATGAATCCGTAGT.

of all of the probes and a thermostable ligase were added to each individual DNA sample. Subsequent to ligation, noncircularized probes and any intermolecular ligation products were removed through exonuclease treatment (Fig. 1). Ligated probes were amplified by PCR with one common primer and two alternative, allele-specific primers labeled with the fluorescent dyes Cy5 and Cy3, respectively (Fig. 1). The resulting fluorescently labeled amplification products were hybridized to a microarray equipped with probes targeting locus-specific tag sequences, and genotypes at individual loci were determined by measuring allele-specific fluorescence signals at the respective positions on the microarray (Fig. 1).

The application of a pool of these probes to chromosomal DNA extracted from *S. aureus* cultures enabled the simultaneous determination of an isolate's phylogenetic affiliation, consistent with MLST, and the presence and type of the methicillin resistance determinant SCC*mec*. Signals at the 16S rRNA gene probes were used for data normalization.

SNP determination. In a series of recent publications, two different sets of highly informative SNPs were recommended that had been selected on the basis of their discriminatory power achieved on the entire MLST database for *S. aureus*, as measured by the Simpson index of diversity (D) (16, 30, 38). This numerical index provides a measure of the probability that any two different sequence types will be discriminated by a given subset of SNPs (15). The two overlapping sets each consisted of seven or eight "high-D SNPs," respectively (i.e., SNPs resulting in a high Simpson index, D), resulting in a total of 11 SNPs (16, 30). Ten of these SNPs we have included in the present study (Tables 3 and 4). The SNP excluded, tpi-241, is virtually invariant among sequences currently in the MLST database (Table 4). In addition, we interrogated three SNPs within MLST loci (tpi-87, tpi-153, and yqiL-505; Tables 3 and 4) that are diagnostic for two MRSA clones that are highly relevant for MRSA epidemiology in central Europe: sequence type ST225 currently is one of the two most frequently isolated

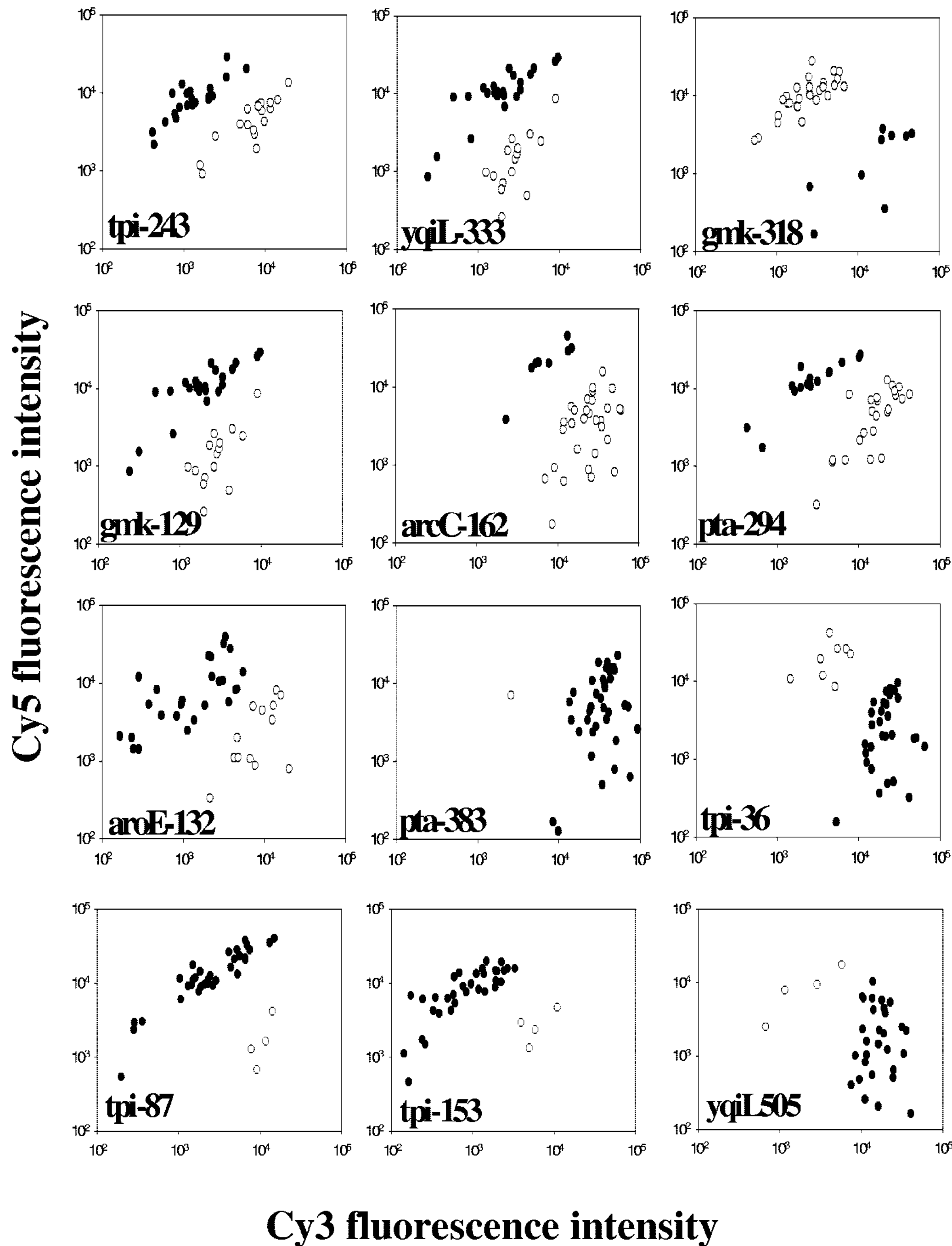

FIG. 2. Scatterplots depicting the fluorescence intensities of the dyes Cy5 and Cy3 for padlock probes targeting SNPs as indicated. Filled and open circles indicate the two different alleles for each of the SNPs as determined by Sanger sequencing. Results are from the 38 isolates listed in Table 1.

MRSA strains in Germany and neighboring countries, and ST228 and descendants (CC228) had been abundant in Germany during the 1990s and are still highly prevalent in Austria and the Balkan states (34, 44).

For SNP determination, pairs of padlock probes were designed to determine the two alleles at each locus (Table 3). When we initially tested the assay on 38 *S. aureus* isolates (Table 1), we achieved 100% typeability (the proportion of isolates that could be genotyped), and the results were fully concordant with Sanger sequencing (Fig. 2). In scatterplots of fluorescence intensities from the dyes Cy5 and Cy3, data points from the different alleles formed clusters (Fig. 2) which could be used to assign genotypes to unknown samples. In subsequent analyses of a limited number of blinded samples (n = 28, Table 2), 100% typeability and 100% accuracy (the proportion of correct results as determined by sequencing) were achieved.

TABLE 4. Allelic profiles

Lineage	Allelic profile													
	arcC-162	arcC-210	aroE-132	gmk-129	gmk-318	pta-294	pta-383	tpi-36	tpi-241[a]	tpi-243	yqiL-333	tpi-153	tpi-87	yqiL-505
CC 1	T	C	A	C	A	A	T	C	G	A	C	T	C	C
CC 5	T	C	A	T	T	A	A	C	G	A	C	T	C	C
ST225	T	C	A	T	T	A	A	C	G	A	C	C	C	C
ST228	T	C	A	T	T	A	A	C	G	A	C	T	A	T
CC 8	T	T	A	C	A	C	A	C	G	A	T	T	C	C
CC 239	A	T	A	C	A	C	A	C	G	A	T	T	C	C
CC 22	T	C	G	C	T	A	A	C	G	G	T	T	C	C
CC 30	A	T	G	T	T	C	A	C	G	G	T	T	C	C
CC 45	A	C	G	T	T	C	A	C	G	G	T	T	C	C
ST617	T	T	G	T	T	C	A	C	G	G	T	T	C	C
CC 59	T	T	A	T	T	C	A	T	G	G	T	T	C	C
CC 80	T	C	A	C	A	C	A	C	G	G	C	T	C	C
CC 121	T	T	G	T	T	C	A	T	G	G	T	T	C	C

[a] SNP tpi-241 was not used for lineage identification.

Since we used a combination of two previously recommended sets of high-D SNPs (16, 30), the 10 SNPs tested may provide partially redundant information. We therefore reevaluated subsets of these SNPs for their in silico performance against the current MLST database by using the Minimum SNPs software (30). Results indicated that the SNPs gmk-129 and pta-383 provide the least information and may be omitted without changing the assay's specificity or discriminatory power. The Simpson index of diversity achieved with the entire database will remain unchanged at 0.95.

At least four independent horizontal transfer events have previously been documented that have led to the exchange of *spa* genes between different clonal complexes of *S. aureus* (31, 39). Obviously, *spa* typing assigns the descendant clones to the lineages of the respective *spa* donor strains, even though the largest parts of the chromosomes have different ancestries, and hence provides misleading results (11, 31, 39). Several example isolates with such mosaic genomes were included in the present study (Tables 1 and 2). Sequence types ST239 and ST241 are related to clonal complex CC8 yet harbor the *spa* and *arcC* genes from CC30 (31); ST617 is affiliated with CC45 and with *spa* and *arcC* derived from CC8 (39), and ST34 is affiliated with CC30 and has the *spa* and *arcC* genes derived from ST10/ST145 (31). Based on high-D SNPs, all of these isolates were correctly identified, demonstrating that the approach is less prone to misclassification following genetic recombination than single-locus sequencing and hence that it may usefully amend *spa* typing.

SCC*mec* genotyping. To identify currently recognized types of SCC*mec*, we applied a set of padlock probes for sequence-specific detection of recombinase genes (Table 3). Gene detection is conceptually different from SNP determination, since there are no built-in positive controls at each individual tag probe on the microarray. Rather, depending on the respective recombinase gene sequence measured, a subset of tag probes will display no fluorescence signal at all. As a consequence, a fluorescence threshold and an interpretation algorithm are needed for positive gene detection. Data interpretation was straightforward, however, since at positively detecting probes, high signal-to-noise ratios were achieved and fluorescence intensities were at least three times stronger than at negative probes (Fig. 3).

We used one padlock probe to detect *mecA*, five padlock probes targeting segments of the *ccrB* genes that are specific for SCC*mec* types I to IV and VI, respectively (21, 28, 29), and one probe targeting the *ccrC* gene, which is the only recombinase gene of SCC*mec* type V (18) (Table 3). When we applied the newly developed assay to 66 *S. aureus* isolates, including 28 blinded samples (Tables 1 and 2), we achieved 100% typeability and 100% accuracy. Our results held two surprises, however.

Firstly, one isolate (93/1000) was indicated to contain both *ccrB* of SCC*mec* type IV and *ccrC* (Fig. 3). While *ccrC* is the only recombinase gene of SCC*mec* type V, it was previously reported to also occur in SCC*mercury*, which is another SCC that carries the mercury resistance determinant *mer* instead of *mecA* and which has frequently been detected in MRSA at a chromosome position directly adjacent to SCC*mec* type III (6). By using a dedicated PCR, we detected the *mer* gene in isolate 93/1000, which, in combination with the detection of *ccrC*, suggests that SCC*mercury* and SCC*mec* type IV may exist simultaneously within the same isolate. It was not investigated if the two putative SCC elements were integrated next to each other at the same chromosome site. In any case, this result confirmed that *ccrC* is not specific for SCC*mec* type V and that its detection together with *mecA* may indicate the presence of SCC*mec* type V only in the absence of other recombinase genes.

Secondly, oxacillin-susceptible isolate 05-01290 unexpectedly was indicated to contain a *ccrB* gene typical of SCC*mec* type I, even though the *mecA* gene was absent (Fig. 3). Posterior PCR analysis not only confirmed the presence of *ccrB* and the absence of *mecA* but further indicated the presence of additional components of an SCC*mec* element in this isolate, including the genes *ccrA*, *ccu*, and *cch* (21). Sequence analyses of these genes confirmed they were indeed derived from an SCC*mec* element of type I (21). However, a PCR assay designed to detect the downstream edge of SCC*mec* elements integrated within the *S. aureus* chromosomal *orfX* gene (14) and PCR-based SCC*mec* classification attempts yielded no

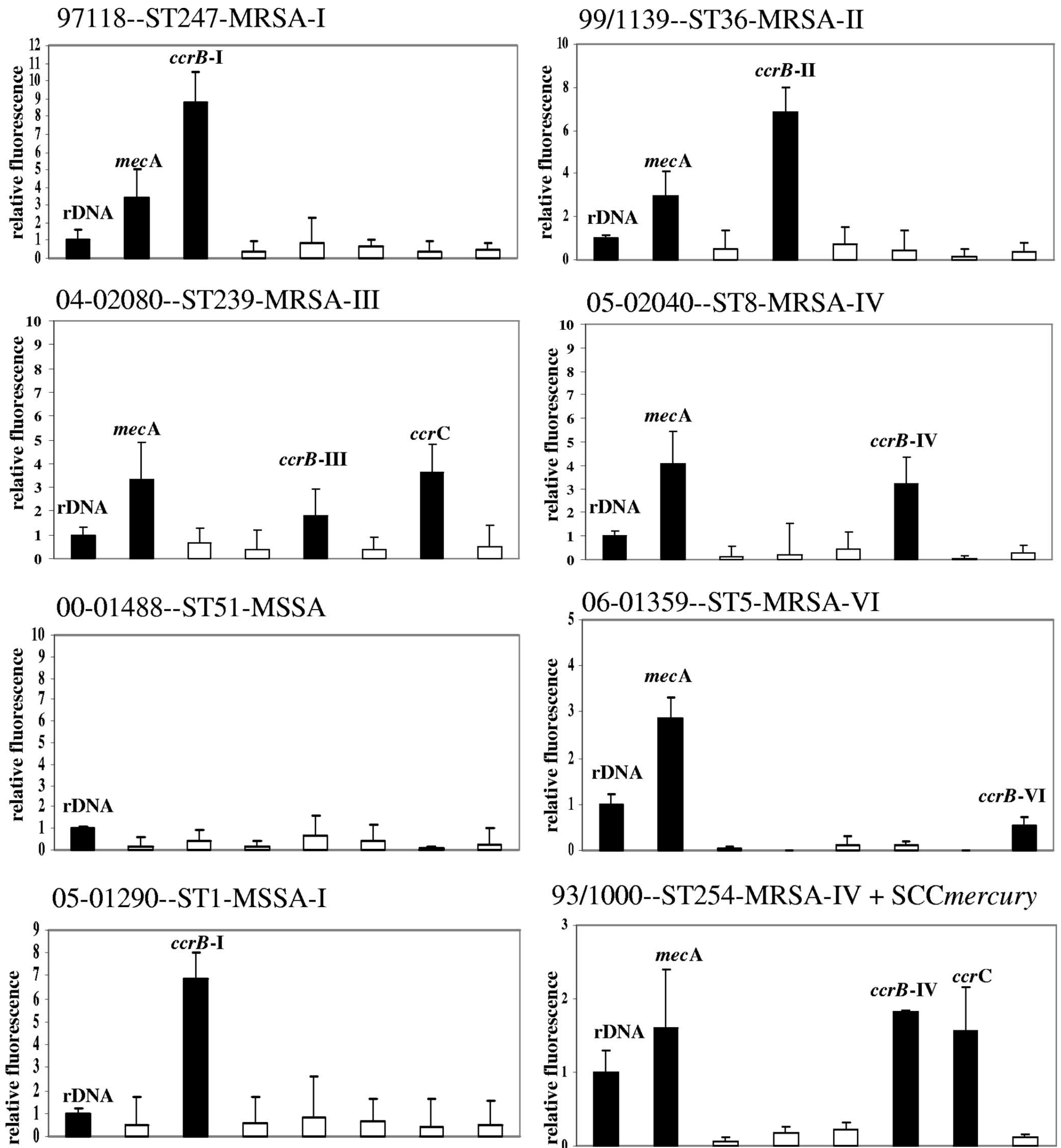

FIG. 3. Examples of gene detection results obtained with eight *S. aureus* isolates. Relative fluorescence intensities (means and standard errors from eight replicate spots) are shown for probes targeting (from left to right) the rRNA gene (rDNA), *mecA*, *ccrB*-I, *ccrB*-II, *ccrB*-III, *ccrB*-IV, *ccrC*, and *ccrB*-VI (roman numerals denote the SCC*mec* types detected). Black bars indicate positive hybridization results. Signals at the rRNA gene probe were used for data normalization.

PCR products. We conclude that this isolate harbors remnants of an SCC*mec* type I element but lacks the methicillin resistance determinant *mecA* and additional segments of the SCC*mec* element. Likely, parts of this strain's SCC*mec* element were deleted either in vivo before the isolate was collected or in vitro during laboratory cultivation or storage (14).

Since genotyping of functional SCC*mec* elements based on padlock probes targeting specific recombinase gene sequences

was fully concordant with PCR-based SCC*mec* genotyping, our results confirm the utility of sequence-based classification of SCC*mec* elements, as independently proposed by two research groups recently (21, 29). Increased discriminatory power may be achieved by an alternative approach based on the detection of SCC*mec* gene content variation (37). However, we preferred a phylogenetic approach for SCC*mec* typing since gene content may vary in a saltatory fashion and in less predictable ways than recombinase gene sequences.

Conclusions and outlook. By using circularizing padlock probes and a microarray equipped with universal tag probes, we were able to combine SNP determination and *mecA* gene and recombinase gene detection in a single assay. As a result, it was possible to simultaneously identify an isolate's phylogenetic affiliation with any of the major clonal lineages or prevalent subclones and its complement and type of SCC*mec* element. This approach will usefully amend *spa* typing as recently proposed (7, 11, 23, 39).

The multiplexing capability of this approach is a major advantage over methods relying on locus-specific PCR. Scale-up of the padlock probe approach to more than 1,000 probes in a single tube was previously demonstrated (12). The assay reported in the present paper may easily be extended with additional probes, which may target, for example, multiple phylogenetically informative polymorphisms that are ascertained through mutation discovery and comparative genome sequencing projects (13, 23, 33, 42). It is expected that such polymorphisms will be highly useful for SNP-based bacterial typing in the near future (3, 23, 25).

To enable increased sample throughput, the homegrown glass arrays that were used in this pilot study, together with manual data acquisition and interpretation, need to be replaced with more sophisticated platforms amenable to extensive automation. While long oligonucleotides are costly, the application of the method will be cost-effective at sufficient sample throughput since minute amounts of reagents are consumed per reaction (1 fmol of each padlock probe) and because universal tag arrays and universal fluorescently labeled oligonucleotide primers are applied, both of which may be combined with multiple different sets of padlock probes.

ACKNOWLEDGMENTS

We are grateful to Annette Weller, Christa Cuny, Mike Henkel, Birgit Pasemann, and the team at our DNA sequencing unit for excellent technical assistance. We thank Phil Giffard for providing the Minimum SNPs software and Johan Stenberg for the Probemaker software.

REFERENCES

1. **Aires-de-Sousa, M., K. Boye, H. de Lencastre, A. Deplano, M. C. Enright, J. Etienne, A. Friedrich, D. Harmsen, A. Holmes, X. W. Huijsdens, A. M. Kearns, A. Mellmann, H. Meugnier, J. K. Rasheed, E. Spalburg, B. Strommenger, M. J. Struelens, F. C. Tenover, J. Thomas, U. Vogel, H. Westh, J. Xu, and W. Witte.** 2006. High interlaboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. J. Clin. Microbiol. **44:**619–621.
2. **Antwerpen, M. H., M. Schellhase, E. Ehrentreich-Förster, F. Bier, W. Witte, and U. Nübel.** 2007. DNA microarray for detection of antibiotic resistance determinants in *Bacillus anthracis* and closely related *Bacillus cereus*. Mol. Cell. Probes **21:**152–160.
3. **Baker, S., K. Holt, E. van de Vosse, P. Roumagnac, S. Whitehead, E. King, P. Ewels, A. Keniry, F. X. Weill, D. Lightfoot, J. T. van Dissel, K. E. Sanderson, J. Farrar, M. Achtman, P. Deloukas, and G. Dougan.** 2008. High-throughput genotyping of *Salmonella enterica* serovar Typhi allowing geographical assignment of haplotypes and pathotypes within an urban district of Jakarta, Indonesia. J. Clin. Microbiol. **46:**1741–1746.
4. **Banér, J., A. Isaksson, E. Waldenstrom, J. Jarvius, U. Landegren, and M. Nilsson.** 2003. Parallel gene analysis with allele-specific padlock probes and tag microarrays. Nucleic Acids Res. **31:**e103.
5. **Cai, Y., F. Kong, Q. Wang, Z. Tong, V. Sintchenko, X. Zeng, and G. L. Gilbert.** 2007. Comparison of single- and multilocus sequence typing and toxin gene profiling for characterization of methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **45:**3302–3308.
6. **Chongtrakool, P., T. Ito, X. X. Ma, Y. Kondo, S. Trakulsomboon, C. Tiensasitorn, M. Jamklang, T. Chavalit, J. H. Song, and K. Hiramatsu.** 2006. Staphylococcal cassette chromosome *mec* (SCC*mec*) typing of methicillin-resistant *Staphylococcus aureus* strains isolated in 11 Asian countries: a proposal for a new nomenclature for SCC*mec* elements. Antimicrob. Agents Chemother. **50:**1001–1012.
7. **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.
8. **Enright, M. C., N. P. Day, C. E. Davies, 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.
9. **Feil, E. J., B. C. Li, D. M. Aanensen, W. P. Hanage, and B. G. Spratt.** 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J. Bacteriol. **186:**1518–1530.
10. **Frénay, H. M., A. E. Bunschoten, L. M. Schouls, W. J. van Leeuwen, C. M. Vandenbroucke-Grauls, J. Verhoef, and F. R. Mooi.** 1996. Molecular typing of methicillin-resistant *Staphylococcus aureus* on the basis of protein A gene polymorphism. Eur. J. Clin. Microbiol. Infect. Dis. **15:**60–64.
11. **Hallin, M., A. Deplano, O. Denis, R. De Mendonca, R. De Ryck, and M. J. Struelens.** 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.
12. **Hardenbol, P., J. Banér, M. Jain, M. Nilsson, E. A. Namsaraev, G. A. Karlin-Neumann, H. Fakhrai-Rad, M. Ronaghi, T. D. Willis, U. Landegren, and R. W. Davis.** 2003. Multiplexed genotyping with sequence-tagged molecular inversion probes. Nat. Biotechnol. **21:**673–678.
13. **Holt, K. E., J. Parkhill, C. J. Mazzoni, P. Roumagnac, F.-X. Weill, I. Goodhead, R. Rance, S. Baker, J. M. Duncan, J. Wain, C. Dolecek, M. Achtman, and G. Dougan.** 2008. High-throughput sequencing provides insights into genome variation and evolution in *Salmonella* Typhi. Nat. Genet. **40:**987–993.
14. **Huletsky, A., R. Giroux, V. Rossbach, M. Gagnon, M. Vaillancourt, M. Bernier, F. Gagnon, K. Truchon, M. Bastien, F. J. Picard, A. van Belkum, M. Ouellette, P. H. Roy, and M. G. Bergeron.** 2004. New real-time PCR assay for rapid detection of methicillin-resistant *Staphylococcus aureus* directly from specimens containing a mixture of staphylococci. J. Clin. Microbiol. **42:**1875–1884.
15. **Hunter, P. R., and M. A. Gaston.** 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. **26:**2465–2466.
16. **Huygens, F., J. Inman-Bamber, G. R. Nimmo, W. Munckhof, J. Schooneveldt, B. Harrison, J. A. McMahon, and P. M. Giffard.** 2006. *Staphylococcus aureus* genotyping using novel real-time PCR formats. J. Clin. Microbiol. **44:**3712–3719.
17. **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 in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **45:**1323–1336.
18. **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.
19. **Koreen, L., S. V. Ramaswamy, S. Naidich, I. V. Koreen, G. R. Graff, E. A. Graviss, and B. N. Kreiswirth.** 2005. Comparative sequencing of the serine-aspartate repeat-encoding region of the clumping factor B gene (*clfB*) for resolution within clonal groups of *Staphylococcus aureus*. J. Clin. Microbiol. **43:**3985–3994.
20. **Kuhn, G., P. Francioli, and D. S. Blanc.** 2007. Double-locus sequence typing using *clfB* and *spa*, a fast and simple method for epidemiological typing of methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **45:**54–62.
21. **Lina, G., G. Durand, C. Berchich, B. Short, H. Meugnier, F. Vandenesch, J. Etienne, and M. C. Enright.** 2006. Staphylococcal chromosome cassette evolution in *Staphylococcus aureus* inferred from *ccr* gene complex sequence typing analysis. Clin. Microbiol. Infect. **12:**1175–1184.
22. **Nilsson, M., H. Malmgren, M. Samiotaki, M. Kwiatkowski, B. P.**

Chowdhary, and U. Landegren. 1994. Padlock probes: circularizing oligonucleotides for localized DNA detection. Science **265:**2085–2088.

23. **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. USA **105:**14130–14135.
24. **Nübel, U., P. M. Schmidt, E. Reiß, F. Bier, W. Beyer, and D. Naumann.** 2004. Oligonucleotide microarray for identification of *Bacillus anthracis* based on intergenic transcribed spacers in ribosomal DNA. FEMS Microbiol. Lett. **240:**215–223.
25. **Okinaka, R. T., M. Henrie, K. K. Hill, K. S. Lowery, M. Van Ert, T. Pearson, J. Schupp, J. Schupp, J. Beaudry, S. A. Hofstadler, P. J. Jackson, and P. Keim.** 2008. Single nucleotide polymorphism typing of *Bacillus anthracis* from Sverdlovsk tissue. Emerg. Infect. Dis. **14:**653–656.
26. **Okuma, K., K. Iwakawa, J. D. Turnidge, W. B. Grubb, J. M. Bell, F. G. O'Brien, G. W. Coombs, J. W. Pearman, F. C. Tenover, M. Kapi, C. Tiensasitorn, T. Ito, and K. Hiramatsu.** 2002. Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. J. Clin. Microbiol. **40:**4289–4294.
27. **Oliveira, D. C., and H. de Lencastre.** 2002. Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **46:** 2155–2161.
28. **Oliveira, D. C., C. Milheirico, and H. de Lencastre.** 2006. Redefining a structural variant of staphylococcal cassette chromosome *mec*, SCC*mec* type VI. Antimicrob. Agents Chemother. **50:**3457–3459.
29. **Oliveira, D. C., C. Milheirico, S. Vinga, and H. de Lencastre.** 2006. Assessment of allelic variation in the *ccrAB* locus in methicillin-resistant *Staphylococcus aureus* clones. J. Antimicrob. Chemother. **58:**23–30.
30. **Robertson, G. A., V. Thiruvenkataswamy, H. Shilling, E. P. Price, F. Huygens, F. A. Henskens, and P. M. Giffard.** 2004. Identification and interrogation of highly informative single nucleotide polymorphism sets defined by bacterial multilocus sequence typing databases. J. Med. Microbiol. **53:**35–45.
31. **Robinson, D. A., and M. C. Enright.** 2004. Evolution of *Staphylococcus aureus* by large chromosomal replacements. J. Bacteriol. **186:**1060–1064.
32. **Robinson, D. A., and M. C. Enright.** 2003. Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **47:**3926–3934.
33. **Roumagnac, P., F. X. Weill, C. Dolecek, S. Baker, S. Brisse, N. T. Chinh, T. A. Le, C. J. Acosta, J. Farrar, G. Dougan, and M. Achtman.** 2006. Evolutionary history of *Salmonella* Typhi. Science **314:**1301–1304.
34. **Ruppitsch, W., A. Stöger, O. Braun, B. Strommenger, U. Nübel, G. Wewalka, and F. Allerberger.** 2007. Methicillin-resistant *Staphylococcus aureus*: occurrence of a new *spa* type in two acute care hospitals in Austria. J. Hosp. Infect. **67:**316–322.
35. **Shopsin, B., M. Gomez, M. Waddington, M. Riehman, and B. N. Kreiswirth.** 2000. Use of coagulase gene (*coa*) repeat region nucleotide sequences for typing of methicillin-resistant *Staphylococcus aureus* strains. J. Clin. Microbiol. **38:**3453–3456.
36. **Stenberg, J., M. Nilsson, and U. Landegren.** 2005. ProbeMaker: an extensible framework for design of sets of oligonucleotide probes. BMC Bioinform. **6:**229.
37. **Stephens, A. J., F. Huygens, and P. M. Giffard.** 2007. Systematic derivation of marker sets for staphylococcal cassette chromosome *mec* typing. Antimicrob. Agents Chemother. **51:**2954–2964.
38. **Stephens, A. J., F. Huygens, J. Inman-Bamber, E. P. Price, G. R. Nimmo, J. Schooneveldt, W. Munckhof, and P. M. Giffard.** 2006. Methicillin-resistant *Staphylococcus aureus* genotyping using a small set of polymorphisms. J. Med. Microbiol. **55:**43–51.
39. **Strommenger, B., C. Braulke, D. Heuck, C. Schmidt, B. Pasemann, U. Nübel, and W. Witte.** 2008. *spa* typing of *Staphylococcus aureus* as a frontline tool in epidemiological typing. J. Clin. Microbiol. **46:**574–581.
40. **Strommenger, B., C. Kettlitz, T. Weniger, D. Harmsen, A. Friedrich, and W. Witte.** 2006. Assignment of *Staphylococcus* isolates to groups by *spa* typing, SmaI macrorestriction analysis, and multilocus sequence typing. J. Clin. Microbiol. **44:**2533–2540.
41. **Syvänen, A.-C.** 2005. Toward genome-wide SNP genotyping. Nat. Genet. **37**(Suppl.):S5–S10.
42. **Van Ert, M. N., W. R. Easterday, L. Y. Huynh, R. T. Okinaka, M. E. Hugh-Jones, J. Ravel, S. R. Zanecki, T. Pearson, T. S. Simonson, J. M. U'Ren, S. M. Kachur, R. R. Leadem-Dougherty, S. D. Rhoton, G. Zinser, J. Farlow, P. R. Coker, K. L. Smith, B. Wang, L. J. Kenefic, C. M. Fraser-Liggett, D. M. Wagner, and P. Keim.** 2007. Global genetic population structure of *Bacillus anthracis*. PLoS ONE **2:**e461.
43. **Witte, W., C. Braulke, C. Cuny, B. Strommenger, G. Werner, D. Heuck, U. Jappe, C. Wendt, H. J. Linde, and D. Harmsen.** 2005. Emergence of methicillin-resistant *Staphylococcus aureus* with Panton-Valentine leukocidin genes in central Europe. Eur. J. Clin. Microbiol. Infect. Dis. **24:**1–5.
44. **Witte, W., I. Klare, U. Nübel, B. Strommenger, and G. Werner.** 2008. Emergence and spread of antibiotic-resistant gram-positive bacterial pathogens. Int. J. Med. Microbiol. **298:**365–377.
45. **Witte, W., B. Strommenger, C. Stanek, and C. Cuny.** 2007. Methicillin-resistant *Staphylococcus aureus* ST398 in humans and animals, central Europe. Emerg. Infect. Dis. **13:**255–258.