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Validation of Binary Typing for *Staphylococcus aureus* Strains

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Most of the DNA-based methods for genetic typing of *Staphylococcus aureus* strains generate complex banding patterns. Therefore, we have developed a binary typing procedure involving strain-differentiating DNA probes which were generated on the basis of randomly amplified polymorphic DNA (RAPD) analysis. We present and validate the usefulness of 15 DNA probes, according to generally accepted performance criteria for molecular typing systems. RAPD analysis with multiple primers was performed on 376 *S. aureus* strains of which 97% were methicillin resistant (MRSA). Among the 1,128 RAPD patterns generated, 66 were selected which identified 124 unique DNA fragments. From these amplicons, only 12% turned out to be useful for isolate-specific binary typing. The nature of the RAPD-generated DNA fragments was investigated by partial DNA sequence analysis. Several homologies with known *S. aureus* sequences and with genes from other species were discovered; however, 87% of the probe sequences are of previously unknown origin. The locations of most of the DNA probes on the chromosome of *S. aureus* NCTC 8325 were determined by hybridization. Seven fragments were randomly dispersed along the genome, five were clustered within the 2500- to 2600-kb position of the genome, and the remaining four did not recognize complementary sequences in *S. aureus* NCTC 8325. A total of 103 *S. aureus* strains (69% MRSA) were used for the validation of the binary typing technique. The 15 DNA probes provided stable epidemiological markers, both in vitro (type consistency after serial passages on culture media) and in vivo (comparison of sequential isolates recovered from cases of persistent colonization). The discriminatory power of binary typing ($D = 0.998$) exceeded that of pulsed-field gel electrophoresis ($D = 0.966$) and RAPD analysis ($D = 0.949$). Reproducibility, measured by analyzing multiple strains belonging to a multitude of different epidemiological clusters, was comparable to that of other genotyping techniques used. Contribution of the DNA probes to the discriminatory power of the system was analyzed by comparison of dendrograms. This study demonstrates that binary typing is a robust tool for the genetic typing of *S. aureus* isolates.

Staphylococcus aureus has remained a prime pathogen of nosocomial and community-acquired infections. Worldwide, the increasing prevalence of multiresistant *S. aureus* has become an additional problem (4, 20, 25). Consequently, the epidemiology of *S. aureus* infections needs to be studied, and for this purpose multiple typing techniques based on the detection of DNA polymorphisms have been developed and optimized (3, 22). Nucleotide sequence variations among *S. aureus* strains can be identified by a number of techniques, varying from pulsed-field gel electrophoresis (PFGE) (39, 46) to randomly amplified polymorphic DNA (RAPD) analysis (37). However, these techniques generate complex banding patterns which lack generally accepted interpretation criteria (8, 36). Consequently, comparison of large numbers of fingerprints is very tedious and has little validity beyond the individual laboratory (8, 38, 42). Therefore, we have sought to develop less tedious typing systems that can be interpreted unequivocally. We have identified relatively unique domains within the staphylococcal genome on the basis of RAPD analysis that could be targets for such a typing system. Strain-specific DNA probes which produce a simple binary output were isolated by using hybridization assays. This collection of probes thus constitutes a so-called library typing system that can elucidate genetic polymorphism and clonal relatedness among *S. aureus* strains (45, 46).

In this study, the DNA probes were sequenced and homologies with known sequences and their locations on the physical map of *S. aureus* NCTC 8325 (29) were determined. The performance of this binary typing system was validated by using the evaluation criteria as proposed by Struelens et al. (33), Arbeit (3), and Maslow et al. (22). The performance criteria include the stability, discriminatory power, and reproducibility of the typing system.

MATERIALS AND METHODS

Bacteria. Strains of *S. aureus* ($n = 463$) were pooled from 11 collections previously used for several purposes (Table 1). For cultivation, bacteria from glycerol stocks, stored at −80°C, were inoculated on Columbia III agar (Becton Dickinson, Etten-Leur, The Netherlands) supplemented with 5% sheep blood and incubated at 37°C for 24 h. All strains were identified as *S. aureus* by standard microbiological methods (19). Methicillin resistance was determined by "direct-colony suspension" inoculation of the strains on Mueller Hinton agar (Oxoid CM 337; Brunswig Chemie, Amsterdam, The Netherlands) in the presence of a disk containing 5 μg of methicillin (Oxoid; Brunswig Chemie, Amsterdam, The Netherlands) and after 16 to 18 h of incubation at 35°C. Zone diameters were interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (26).

Binary typing. Binary typing was performed as described previously by van Leeuwen et al. (45, 46). However, we increased the overall number of strain-specific DNA probes from 5 to 15. The same procedures were used for the generation of the DNA probes as described before (45).

(i) Generation of the strain-specific DNA probes. In short, after RAPD analysis DNA fingerprints were compared visually, and unique, strain-differentiating amplicons were selected and subsequently cloned into a TA cloning vector (Invitrogen, Leek, The Netherlands) and then transformed into *Escherichia coli* JM 109 cells. Inserts were amplified from the recombinant plasmids with M13 and T7 primers. Cloned fragments were characterized by DNA sequencing with

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TABLE 1. Characterization of the *S. aureus* collections used in this study

Collection	Collection code(s)[a]	Geographic origin[b]	No. of strains	Original purpose for strain collection in this study	Description of collections	Reference(s) or source
1		United States (CDC)[a]	59		Multicenter collection of MRSA (63%) and MSSA (37%) strains	35
2		Portugal	184		Nationwide disseminated MRSA strains from hospitals	1
3		Worldwide	66	Generation of strain-specific DNA probes	Worldwide collection of MRSA strains	20
4		Italy	49		Genetically unrelated MRSA strains (determined by PFGE), isolated from hospitalized patients (five centers), Sicily, Italy	41
5		Australia	18		Genetically unrelated MRSA strains (determined by PFGE), obtained from four different hospitals	41
6	K2	United States	26	Determination of discriminatory power of the genotyping methods (see Table 4)	Community-acquired MRSA strains	46
7	SA, SB, SC	United States (CDC)	14		Selection of geographically diverse strains from collection 1	35
8	NC	The Netherlands	10	Stability experiment for binary typing probes (see Table 3)	Strains isolated from persistant nasal carriers	43
9	RIVM	The Netherlands	2		Selection of 1 MRSA strain (Va) and 1 MSSA strain (Ia), from collection no. 10	
10	RIVM	The Netherlands	49	Epidemiological applications of the diverse genotyping systems (see Table 5)	MRSA and MSSA strains from 10 outbreaks in 10 Dutch hospitals	This study
11		United Kingdom (NCTC)	2	Mapping of the strain-specific DNA probes (see Fig. 1)	*S. aureus* NCTC 8325 and 8325-4	27, 29

[a] K2, community-acquired MRSA strains from a New York City hospital; SA, SB, SC, *S. aureus* strains from the CDC collection; NC, nasal carrier; RIVM, National Institute of Public Health and the Environment (Bilthoven, The Netherlands).

[b] CDC, Centers for Disease Control and Prevention; NCTC, National Collection of Type Cultures (Central Public Health Laboratory, London, United Kingdom).

dye-terminator chemistry by using a 373 DNA sequencing system (Perkin-Elmer, Foster City, Calif.). The insert sequences were compared with all entries in the data bank of the National Center for Biotechnology Information (NCBI) and were analyzed for nucleotide and protein sequence similarities with Basic Local Alignment Search Tool (BLASTN and BLASTP, respectively [2]).

(ii) Implementation of the binary typing system. Labeling, hybridization, and detection of the cloned DNA fragments were performed with enhanced chemiluminescence (ECL) direct labeling and detection systems, according to the manufacturer's protocols (Amersham Life Science, Buckinghamshire, United Kingdom), in order to use them as probes. The hybridization characteristics of the DNA probes were defined by prescreening these probes on a Southern blot containing 14 genetically unrelated staphylococcal strains (Table 1, collection 7). DNA probes displaying differential hybridization were added to the binary typing system. Hybridization of the 15 different DNA probes was scored with a 1 or a 0 according to the presence or absence of the hybridization signal, respectively, and the resulting binary code was transformed into a decimal number. This number is further represented as the binary type.

RAPD analysis. RAPD analysis was carried out essentially as described before (37). Fingerprints were scored visually in which a single band difference defined a novel RAPD type. The three-letter codes are based on ERIC-2, AP-1, and AP-7 priming (45) and can only be compared within each group and not across the different groups of organisms represented in Tables 3, 4, and 5.

PFGE. Restriction with *Sma*I (Boehringer, Mannheim, Germany) of genomic staphylococcal DNA and subsequent separation of the DNA macrorestriction fragments was performed by contour-clamped homogeneous electric field (CHEF) PFGE as described before (39). Macrorestriction profiles were interpreted as described by Tenover et al. (36), and each pattern is presented as a roman letter.

***Mec-A*–Tn*554* probe typing.** Genomic staphylococcal DNA was digested with *Cla*I endonuclease (Pharmacia Biotech, Roosendaal, The Netherlands) according to the manufacturer's instructions. Generation of target-specific probes and hybridization was done as described before (20).

Coagulase gene PCR. Coagulase gene polymorphism was determined by PCR as described previously (32). The amplified part of the coagulase gene was digested with the restriction endonuclease *Alu*I (Boehringer) according to the manufacturer's protocol. Restriction fragment length polymorphism (RFLP) patterns were visually interpreted and indexed by roman lettering.

PCR analysis of the *mec* regulator genes *mecI* and *mecR1*. PCR was performed as described before (34). Three sets of specific primers were used to amplify the different regions of the *mec* regulator genes, i.e., *mecI* and the 5′ end (transmembrane part) and the 3′ end (penicillin-binding part) of *mecR1*.

***cna* probe.** The presence or absence of the *S. aureus* collagen adhesin (*cna*) was used as an additional genotypic marker for the differentiation of *S. aureus* strains. Probing was performed essentially as described by Smeltzer et al. (31).

***spa* gene.** Staphylococcal protein A (Spa) gene polymorphism was determined by PCR as described previously (11). The so-called X region, a repetitive part within the gene, was amplified, and subsequently, the amplicon was digested with the restriction endonuclease *Rsa*I (Boehringer), resulting in two fragments composed of 214 and 35 bases and a third fragment containing the repetitive DNA. The number of 24-bp repeats was calculated by comparison with a 100-bp molecular weight marker (Pharmacia Biotech).

Phage typing. Phage typing was performed at the Dutch National Institute of Public Health and the Environment by using the international set of typing phages and a set of typical Dutch phages (28, 47). Different phage patterns were given different type designations.

Ribotyping. Conventional ribotyping with *Eco*RI was performed by methods described previously (13). Restriction fragments were Southern blotted onto Hybond N^+ membranes (Amersham) (30), and the *S. aureus* 16S rRNA gene, amplified by PCR, was used as a probe. Hybridization was detected by using an ECL kit (Amersham).

TABLE 2. Summary of the demographic data and the sequence homologies from the RAPD-generated *S. aureus* DNA fragments with strain-specific characteristics ($n = 15$)

Probe code	Origin	Source strain code	Mc result[b]	Probe size (bp)	Specificity[c]	Nucleotide sequence homology[d]		Protein sequence homology[d]	
						M13	T7	M13	T7
AW-1	CDC[a]	SA-08	R	1,200	13	NH	NH	NH	NH
AW-2	CDC	SA-01	R	600	8	NH	NH	NH	NH
AW-3	CDC	SA-06	I	550	7	NH	NH	HrmA	NH
AW-4	CDC	SA-02	R	700	6	*S. aureus* plasmid pSH6	*S. aureus* plasmid pSH6	*S. aureus* transposase IS*257*	*S. aureus* transposase IS*257*
AW-5	Portugal	HPV107	R	400	3	NH	NH	Lysostaphin precursor of *S. simulans*	NH
AW-6	United States	BK1591	R	350	5	NH	NH	NH	NH
AW-7	Italy	246D	R	1,200	4	NH	NH	NH	NH
AW-8	Australia	WBG8217	R	350	5	*B. subtilis yqeV*	*B. subtilis yqeV*	*B. subtilis* hypothetical protein YqeV	*B. subtilis* hypothetical protein YqeV
AW-9	United States	BK1457	R	1,500	5	NH	NH	NH	NH
AW-10	United States	BK1461	R	400	6	NH	NH	NH	NH
AW-11	Australia	WBG8231	R	1,500	6	NH	NH	NH	NH
AW-12	CDC	SB-18	R	350	4	NH	NH	NH	NH
AW-13	Italy	85CCH	R	350	4	NH	NH	NH	NH
AW-14	United States	BK1563	R	650	4	NH	NH	NH	NH
AW-15	Italy	76CCH	R	300	5	NH	NH	NH	NH

[a] CDC, Center for Disease Control and Prevention.
[b] Mc; methicillin susceptibility disk-diffusion test; R, resistant; I, intermediate.
[c] Number of strains hybridizing with a particular probe. The probes were tested on 14 CDC strains (Table 1, collection 7).
[d] NH; no homology. Cloned DNA sequences of insert termini (M13 and T7) were analyzed for sequence homologies with nucleotide and protein sequences in the NCBI data bank by using the BLAST computer program (2).

***MecA* PCR.** All *S. aureus* strains were investigated for the presence of the *mecA* gene by PCR as described before (24).

Physical mapping. Genomic DNAs from *S. aureus* NCTC 8325 (29) and 8325-4 (27) were digested with *Sma*I (Boehringer), *Sgr*AI (Boehringer Mannheim), and *Asc*I (New England Biolabs, Leusden, The Netherlands) according to the manufacturers' protocols. Macrorestriction fragments were separated by PFGE and subsequently transferred onto Hybond N^+ membranes (Amersham) for Southern hybridization (30). Probing with the 15 strain-specific DNA fragments was done as described above under "Binary typing."

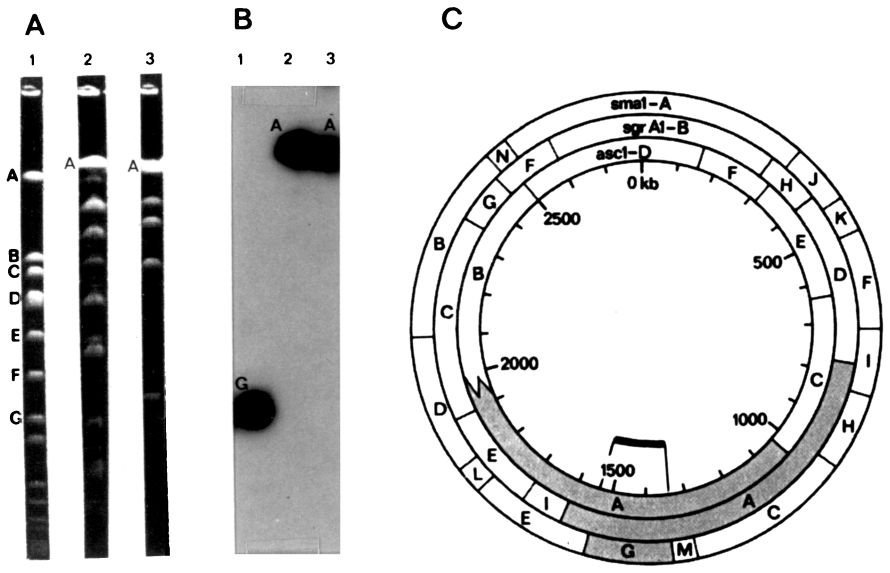
Statistical analysis. The discriminatory power of binary typing and other genotyping formats used in this study, defined as the average probability that different genotypes will be assigned to two unrelated strains in the population of a given genus, was calculated by using the formula of the Simpson index of diversity as explained by Hunter and Gaston (17, 18). The contribution of the DNA probes to the discriminatory power of the binary typing system was analyzed by cluster analysis and comparison of the dendrograms. First, all of the probes ($n = 15$) were used to characterize 40 unique (Table 1, collections 6 and 7) and 10 outbreak clusters (Table 1, collection 10) of *S. aureus* strains. The percentages of similarity of the hybridization patterns were calculated with Dice coefficient and with unweighted pair group mathematical analysis to display relatedness hierarchies among the strains. Subsequently, the procedure was repeated after discarding the DNA probe that had the lowest level of discrimination.

RESULTS

Selection of the strain-specific DNA probes. RAPD analysis with multiple primers was performed on 376 *S. aureus* strains (Table 1, collections 1 through 5) of which 97% were methicillin-resistant. One hundred and twenty-four amplicons were selected from 66 RAPD patterns. Overall, 98 DNA fragments (79%) were successfully cloned, and from those a total number of 17 clones displayed a strain-specific character after hybridization with *Eco*RI-digested DNA from the 14 epidemiologically unrelated *S. aureus* strains (Table 1, collection 7 [38% MRSA]). However, 3 of the 17 clones shared the same DNA sequence, and two of these were consequently discarded. The remaining 80 fragments hybridized with DNA of all strains either at single ($n = 42$) or multiple sites ($n = 14$) or recognized the digested DNA from their source strain ($n = 24$) only. The latter fragments were not included since these fragments did not contribute significantly to the discriminatory power of the system.

Characterization of the DNA probes. The origin and the nature of the 15 RAPD-generated DNA probes are outlined in Table 2. Sequence data were obtained from both termini (M13 and T7), and the DNA sequences were analyzed separately for homology by using the BLAST program with the nucleotide and protein sequence data bank, including the unfinished microbial genomes data bank (NCBI). A large proportion of these sequences did not match with known DNA elements (87%) for the nucleotide sequence data bank and 80% for the protein data bank). Probe AW-3 (M13 terminus) appeared to have a low score (BLAST score of 36) with the gene product encoded by *hrmA* of *Nostoc* sp. in the protein sequence database. Probe AW-4 (both termini) displayed a high score (1571) with the *S. aureus* multiresistance plasmid pSH6 for insertion sequences IS*256* and IS*257* in the search of the nucleotide sequence data bank and a high score (593) with IS*257* transposase in the search of the protein sequence data bank. The M13 terminus of probe AW-5 displayed a low homology score (89) with the lysostaphin precursor of *Staphylococcus simulans*. Finally, probe AW-8 (both termini) appeared to have a high level of similarity (BLAST score 573) with the *yqeV* gene, a hypothetical protein, and part of the polycystronic locus of the *Bacillus subtilis dnaK* operon.

The locations of the strain-specific DNA probes were determined on the physical map of the *S. aureus* NCTC 8325 genome (Fig. 1) and on the restriction fragments of *S. aureus* NCTC 8325-4. Four of the 15 DNA probes (AW-1, AW-2, AW-4, and AW-7) failed to hybridize to either of the two staphylococcal genomes. Five probes (AW-6, AW-10, AW-12, AW-13, and AW-14) were found to be physically clustered in the same DNA region (position 2500 to 2600 kb), while the



DNA probe	Hybridization to fragment		
	Sma-1	*Sgr-A1*	*Asc-1*
AW-1	NH	NH	NH
AW-2	NH	NH	NH
AW-3	C	A	A
AW-4	NH	NH	NH
AW-5	C	A	C
AW-6	A	F	D
AW-7	NH	NH	NH
AW-8	A	H	E
AW-9	B	C	B
AW-10	A	F	D
AW-11	E	A	A
AW-12	A	F	D
AW-13	A	F	D
AW-14	A	F	D
AW-15	**G**	**A**	**A**

FIG. 1. Physical mapping of the DNA probes on *S. aureus* NCTC 8325 (29) restriction fragments. (A) PFGE macrorestriction patterns of *S. aureus* NCTC 8325 digested with *Sma*I, *Sgr*AI, and *Asc*I (lanes 1, 2, and 3, respectively). Restriction fragments are coded by descending molecular size. (B) Example of hybridization results with probe AW-15 to PFGE patterns of *S. aureus* NCTC 8325. Lanes are the same as for panel A. (C) Hybridization results of probe AW-15 depicted on the physical map of *S. aureus* NCTC 8325. (D) Mapping results of the 15 strain-specific DNA probes (AW-1 through AW-15) to the macro-restriction fragments of the *S. aureus* NCTC 8325 genome. NH, no hybridization of the strain-specific DNA probe to the macrorestriction fragments.

remaining seven probes were found to be scattered on the physical map of *S. aureus* NCTC 8325.

Stability experiments. The in vivo stability of the binary typing system was assessed by testing sequential isolates of *S. aureus* from five individuals who were previously classified as being persistent nasal carriers (Table 1, collection 8) (43). The 15 DNA probes uniformly and correctly identified each of the two *S. aureus* strains isolated from five persistent nasal carriers in 1988 and 1995, respectively, in accordance with the other genotyping techniques (Table 3). Moreover, we tested the in vitro stability of the DNA probes by serial passage (50×) of strains Ia and Va (Table 1, collection 9). Again, all descendent isolates were shown to be identical, i.e., their binary types did not change with serial passages (data not shown).

Discriminatory power. We compared the discriminatory power of our binary typing system with that of generally accepted typing systems included PFGE, RAPD analysis, and *mecA*-Tn*554* probing. Comparative analysis of the discriminatory power of these genotyping systems is displayed in Table 4 and is expressed by the Simpson index of diversity (*D*). RFLP analysis of the *mecA* gene and Tn*554* generated 11 unique patterns from the 40 epidemiologically unrelated strains (Table 1, collections 6 and 7) and had the lowest score ($D = 0.848$). Due to the absence of the *mecA* gene, the typing data of the MSSA strains were deleted for the *D* value determination. PFGE and RAPD analysis differentiated the collection into 25 and 19 subtypes with *D* values of 0.966 and 0.949, respectively. However, the binary typing system distinguished 38 unique genotypes and had a *D* score of 0.998. Only two binary types, 31969 and 31647, were each found twice in the collection. Type 31647 was found to be identical by PFGE, RAPD analysis, and *mecA* gene polymorphism, but one strain

TABLE 3. Stability of the strain-differentiating DNA probes determined with *S. aureus* strains obtained from persistent nasal carriers

Persistent carrier (sex)[a]	Strain[b]	Isolation date	Binary code (type)[c]	Result by indicated genotyping technique[d]:				
				RAPD analysis	PFGE	*spa* polymorphism	Coagulase gene polymorphism	Presence/absence of *cna*
099 (F)	NC-711	1988	001010110101111 (5551)	CCC	J	9	F	−
	NC-220	September 1995	001010110101111 (5551)	CCC	J	9	F	−
060 (F)	NC-1740	1988	101010011100101 (21733)	FCC	M	9	D	−
	NC-1733	February 1995	101010011100101 (21733)	FCC	M	5	D	−
038 (F)	NC-1288	1988	000000011100111 (231)	EDE	G	7	B	−
	NC-105	June 1995	000000011100111 (231)	EDE	G	7	B	−
076 (F)	NC-705	1988	000010011100101 (1253)	AEA	A	7	A	+
	NC-054	May 1995	000010011100101 (1253)	AEA	A	7	A	+
145 (M)	NC-714	1988	000000010100101 (165)	BBB	B	8	B	+
	NC-063	May 1995	000000010100101 (165)	BBB	B	8	B	+

[a] Persistency defined as 10 identical culture results from longitudinal sampling over 3 months (43).
[b] NC, strain from nasal carrier. See Table 1, collection 8, for more details.
[c] Overall results after hybridization with 15 strain-specific DNA probes (AW-1 through AW-15, respectively). The decimal number represents the binary type. See Materials and Methods for more information.
[d] Results (except for presence/absence of *cna*) are given as codes for each technique. See Materials and Methods for more information.

TABLE 4. Analysis of the discriminatory power of the binary typing method compared with other genotyping techniques, estimated on the basis of the typing results for epidemiologically unrelated MRSA strains from New York City and geographically diverse *S. aureus* strains from the United States[a]

Strain	Binary code	Binary type	Result by indicated genotyping technique			
			PFGE	RAPD analyses	*mecA*-Tn*554* probing	Methicillin resistance[b]
K2-01	001110111110111	7671	J	HMC	IV:M	R
K2-02	110001011100101	25317	K	AAK	II:F	R
K2-06	001110111100111	7655	L	NHB	III:NH	R
K2-07	000100100100111	2343	M	CCC	II:NH	R
K2-12	001110100100111	7463	J	HMC	IV:M	R
K2-13	101110111110101	24053	M	NBB	II:NH	R
K2-19	001100100100101	6437	J	MMC	IV:M	R
K2-20	011011111100001	14305	N	NHB	II:NH	R
K2-21	111110011100001	31969	O	NBB	II:NH	R
K2-22	000010111111111	1535	P	BBB	I:unique	R
K2-24	001100000110111	6199	Q	NNA	I:NH	R
K2-30	001110100110011	7475	J	GOC	I:A	R
K2-31	011111110111101	16317	R	NHB	II:NH	R
K2-32	001110100111111	7487	J	HMC	IV:M	R
K2-34	011110111111101	15869	S	NHB	II:NH	R
K2-38	011110111110101	15861	T	BIB	I:A	R
K2-40	011110111110001	15857	T	BBB	I:A	R
K2-44	001100101110011	6515	U	MNA	I:E	R
K2-45	001110001100011	7267	J	HMC	IV:M	R
K2-47	011110011100001	15585	V	NHB	II:NH	R
K2-50	001111111101111	8175	V	BBB	I:A	R
K2-51	101111111111101	24573	V	BBB	I:A	R
K2-52	001111010110111	7863	J	HMC	IV:M	R
K2-56	011110011110011	15603	W	HMC	Unique:M	R
K2-57	000100110111101	2493	X	JAE	I:D	R
K2-65	101110110100011	23971	Y	PPH	Unique:E	R
SA-04	111010000000000	29696	E	FSL	NH:X	S
SA-06	101110000100000	23584	C	TTM	II:NH	I
SA-07	111110011100000	31968	B	TTM	NH:NH	S
SA-08	111110011100001	31969	G	VSL_1	I:NH	R
SA-11	100100001000000	18496	F	VSL_1	II:NH	R
SA-12	111110001100000	31840	A	TTM	I:A	R
SA-13	111110000110000	31792	A	TTM	I:A	R
SA-14	100100000100000	18464	H	TTM_1	NH:NH	S
SA-17	111110000010000	31760	A	TTM	I:A	R
SB-02	000001101011111	863	I	WUN	NH:NH	S
SB-09	111101110011111	31647	I	WVN	NH:Z	S
SB-13	111101110011111	31647	I	WVN	NH:NH	S
SC-06	101111110000111	24455	Z	JXO	NH:NH	S
SC-08	100000000011000	16408	Z	JXO	NH:NH	S
Total number of types		38	25	19	11[c]	3
D		0.998	0.96	0.949	0.848	

[a] See Table 1 for more information on the origins of the strains used.
[b] R, resistant; I, intermediately resistant; S, susceptible.
[c] The *mecA*-Tn*554* typing data for the MSSA strains ($n = 8$) were deleted.

(SB-13) lacked Tn*554*. Types 31969 and 31647 reportedly also share a single phage type (35). Strains K2-21 and SA-08 share binary type 31969 but clearly differed by the other genotyping systems (Table 4). It has to be emphasized that a common clonal origin for some of the (even epidemiologically unrelated) strains described in the present communication cannot be fully excluded.

Reproducibility. In order to test the reproducibility of our binary typing system, we tested 10 different clusters of epidemiologically related *S. aureus* strains (four to five strains per outbreak; four MSSA and six MRSA clusters). A representative illustration of the binary typing hybridization results is outlined in Fig. 2. The genetic relatedness of the strains within a cluster was primarily defined on the basis of epidemiological data and possession of identical phage types within the cluster. The reproducibility of the binary typing technique was calculated as the number of isolates correctly assigned to the same type within a cluster divided by the total number of strains tested. Overall, 45 of 49 (92%) strains were correctly typed, i.e., 30 of 30 MRSA and 15 of 19 MSSA strains (Table 5). Interestingly, the nonconcordant MSSA strains also showed genetic variation by one or more of the other genotyping systems applied to the same set of strains. Thus, binary typing is similarly sensitive to such variation in the genome of *S. aureus*.

The contribution of the DNA probes to the discriminatory power of the system was analyzed by comparison of the dendrograms. Increasing subtraction of the distinct DNA probes reduced the resolution of the binary typing system among the

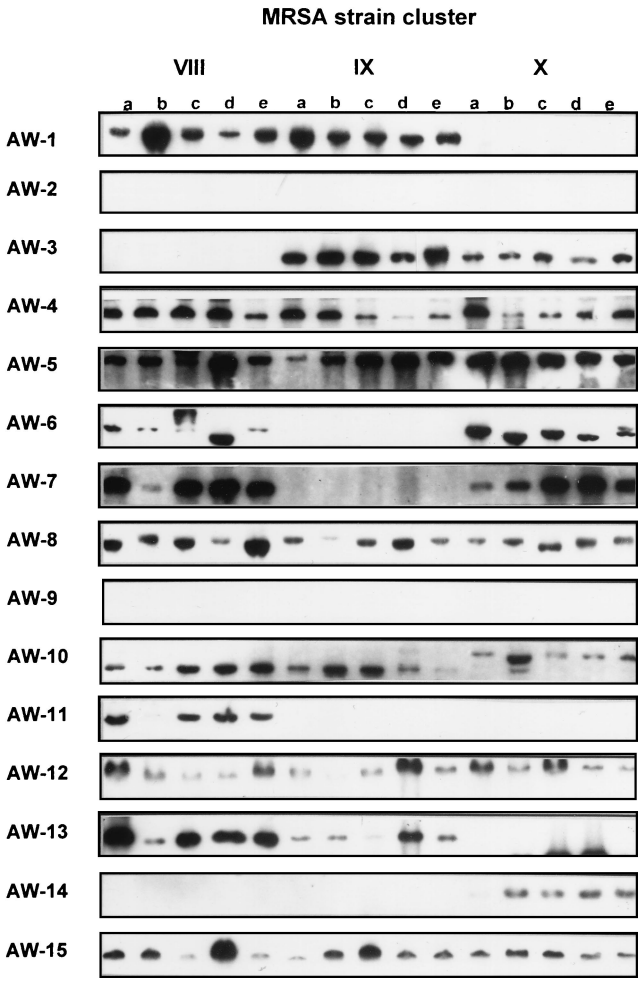


FIG. 2. Representative example of binary typing results obtained with the complete panel of strain-specific DNA probes (AW-1 through AW-15) from three MRSA clusters VIII, IX, and X (n = 15, collection 10). Each cluster encompassed five strains (a, b, c, d, and e) and displayed identical hybridization results.

genotypic results of the unique *S. aureus* strains (Fig. 3a). A similar effect on the epidemiological concordance among the hybridization patterns of the *S. aureus* outbreak cluster strains was noticed after subtraction of the same DNA probes (Fig. 3b). Consequently, none of the probes can be discarded from the binary typing system.

DISCUSSION

Whole genomes of bacteria are currently being sequenced at high rates, and information can be derived from analysis and comparison of these chromosomes. Essential paralogous regions as well as narrowly distributed gene families can be identified. The latter groups may be genus, species, or even strain specific. For instance, the genome of *Mycoplasma genitalium* commits about 5% of its content to a single species-specific domain, encoding an adhesin gene (10). Another type of DNA variability was observed after completion of the *Haemophilus influenzae* DNA sequence (9). Repeats in the genes encoding enzymes involved in lipopolysaccharide biosynthesis and iron acquisition and a gene encoding an adhesin display clear heterogeneity (16, 40). The *E. coli* genome highlights novel insertion sequence elements, phage remnants, and many DNA fragments of unusual composition, indicating genome plasticity and horizontal gene transfer (5). Many bacterial virulence genes are found as discrete DNA fragments, present in pathogenic organisms but absent from nonpathogenic members of the same genus or species, e.g., the "pathogenicity islands" of uropathogenic *E. coli* or enteropathogenic *E. coli* (14, 23). Unfortunately, only a single genome sequence of a gram-positive bacterium is known. The *B. subtilis* genome contains phage-type elements as well, again indicating DNA flexibility (21). Based on theoretical comparative analysis, many DNA elements contributing to DNA variation can be pinpointed. No experimental studies have been described as yet, however. Practically, the genome variability of *S. aureus* strains can be visualized on the basis of RAPD analysis and the use of the amplicons thereof as probes. We describe here an approach for isolating species-specific DNA elements for a bacterium for which the whole genome sequence is not in the public domain. The aim of the present study was to validate the use of strain-differentiating DNA probes for the genotyping of *S. aureus* and to develop a new typing format, providing a simple binary output based on the use of RAPD-generated DNA probes. Such probes can detect sequence variation between genomes without prior knowledge of the target DNA sequence, as has been presented before (45, 46). We now have extended the number of DNA probes to 15 and have shown the typing system to have a very high index of reproducibility, stability (100%), and discriminatory power (D = 0.998). Hybridization studies revealed that only 12% of the RAPD amplicons, visually selected for uniqueness, exhibited the desired genetic typing characteristics for *S. aureus* strains. Primer site variation may be the origin of the remaining 88% of the differentiating amplicons. The nature of the DNA probes used in this study remains largely unknown. In one case (probe AW-4) homology with a mobile genetic element, IS*257*, was found. IS*257* is an insertion sequence identified as commonly occurring in staphylococcal plasmids (7). These plasmids often code for diverse resistance determinants. The investigation of further alignments awaits publication of the whole *S. aureus* genome sequence.

The locations of the DNA probes on the physical map of *S. aureus* NCTC 8325 (29) (Fig. 1) and *S. aureus* NCTC 8325-4, a derivative of 8325 cured of phages P11, P12, and P13 (27), were determined. Some probes (n = 4) were neither on the physical map of S. aureus NCTC 8325 nor present on the restriction fragments of NCTC 8325-4. The remainder of the probes recognized elements on both genomes, which argues against a putative relationship with the prophage sequences that are present in *S. aureus* NCTC 8325 but not in *S. aureus* NCTC 8325-4. Seven probes showed random locations and five clustered together around the 2500- to 2600-kb region of the *S. aureus* NCTC 8325 genome. These latter probes all share a nucleotide sequence of 80 bp, but the main part of their nucleotide sequence was totally different. It is possible that this DNA region is part of a direct repeat and spacer region, which can be used to generate sequence variation patterns between genomes (44). The location of these probes coincides with that of several potentially variable elements: essential genes for recombination between genomes (*recA*) or DNA repair (*uvr*), virulence factors (*hla*), and diverse Tn*551* insertion sites (29). The probes that hybridized to DNA regions scattered throughout the genome seemed to have no linkage with variable DNA sequences, except for one probe (AW-15) which is located in the vicinity of resistance determinants (*mec* region), virulence factors (*spa*), and the origin of replication.

The stability of the binary typing system was evaluated with

TABLE 5. Survey of geno- and phenotypic results for epidemic outbreak strains of MRSA and MSSA from Dutch hospitals and nursing homes

Cluster code[a]	Epidemiological data			Isolation date (mo/yr)	Binary code[b]	Binary type[c]	Phage type[d]	Ribo-type[e]	Result for indicated typing method:								
	Hospital	Patient	Isolate source						PFGE[f]	RAPD analysis[g]	Coagulase gene polymorphism[h]	*cna*[i]	*spa*[j]	*mecA*[k]	*mecI*[k]	*mecR1*[k] 5′	*mecR1*[k] 3′
MSSA																	
Ia	A	1	Groin	11-96	101001111111101	21501		A	A	AAA		−/−	11	−	−	+	−
Ib	A	1	Sputum	11-96	101001111111101	21501		A	A	AAA	A	−/−	11	−	−	+	−
Ic	A	1	Blood	11-96	101001111111101	21501		A	A	AAA	A	−/−	11	−	−	+	−
Id	A	2	Sputum	11-96	101001111111101	21501		A	A	AAA	A	−/−	11	−	−	+	−
Ie	A	3	Blood	07-96	101001111111101	21501		A	A	AAA	A	−/−	11	−	−	+	−
IIa	B2	1	Pus	12-96	001000000100000	4128		A	B	BBB	NR	+/+	5	−	−	+	−
IIb	B1	1	Pus	10-96	001000000100000	4128		A	B	B_1BB	NR	+/+	5	−	−	+	−
IIc	B1	2	Pus	08-96	001000000100000	4128		A	B_1	B_1BB	NR	+/+	10	−	−	+	−
IId	B2	3	Pus	11-96	001000010100000	4256		A	B_2	B_1BB	NR	+/+	11	−	−	+	−
IIe	B2	4	Pus	10-96	001000000100000	4128		A	B	BBB	NR	+/+	5	−	−	+	−
IIIa	B1	5	Pus	11-96	000000110101011	427		B	C	CCC	NR	−/−	10	−	−	+	−
IIIb	B3	6	Urine	10-96	000000110101011	427		B	C	CCC	NR	−/−	10	−	−	+	−
IIIc	B3	6	Urine	10-96	000000110101011	427		B	C	CCC	NR	−/−	10	−	−	+	−
IIId	B3 (B1)[l]	7	Pus	12-95	010000110101011	8619		B	C_1	CCC	NR	−/−	10	−	−	+	−
IIIe	B1	8	Urine	02-96	010000110101011	8619		B	C_1	CCC	NR	−/−	10	−	−	+	−
IVa	C	1	Nose	11-96	001011010100010	5794		A	D	DDD	B	−/+	7	−	−	+	−
IVb	D (C)[m]	2	Exit site	04-96	001001010100010	4770		A	D	DDD	B	+/+	9	−	−	+	−
IVc	C	3	Nose	07-96	001011010100010	5794		A	D_1	DDD_1	B	−/+	11	−	−	+	−
IVd	C	4	Pus	08-96	001011010100010	5794		A	D_1	DDD_1	B	−/+	11	−	−	+	−
MRSA																	
Va	E[n]	1	Unknown	01-96	110111111111111	28671	XVI-3	A	E_1	EEE_1	C	+/+	10	+	+	+	+
Vb	E	2	Unknown	01-96	110111111111111	28671	XVI-3	A	E_1	EEE_1	C	+/+	10	+	+	+	+
Vc	E	3	Unknown	01-96	110111111111111	28671	XVI-3	A	E_1	EEE_1	C	+/+	10	+	+	+	+
Vd	E	4	Unknown	01-96	110111111111111	28671	XVI-3	A	E	EEE_1	C	+/+	10	+	+	+	+
Ve	E	5	Unknown	01-96	110111111111111	28671	XVI-3	A	E_1	EEE_1	C	+/+	10	+	+	+	+
VIa	F1[o]	1	Unknown	10-96	001110010000000	7296	XVI-4	A	F	FFB	NR	+/+	9	+	−	+	−
VIb	F1	2	Unknown	05-96	001110010000000	7296	XVI-4	A	F	FFB	NR	+/+	9	+	−	+	−
VIc	F1	3	Nose	05-96	001110010000000	7296	XVI-4	A	F	FFB	NR	+/+	9	+	−	+	−
VId	F1	4	Unknown	07-96	001110010000000	7296	XVI-4	A	F	FFB	NR	+/+	9	+	−	+	−
VIe	F1	5	Unknown	04-96	001110010000000	7296	XVI-4	A	F	FFB	NR	+/+	9	+	−	+	−
VIIa	F2[o]	1	Nose	10-96	001110000000000	7168	XI-6	A	F_1	FFB	NR	+/+	9	+	−	+	−
VIIb	F2	2	Nose	10-96	001110000000000	7168	XI-6	A	F_1	FFB	NR	+/+	9	+	−	+	−
VIIc	F2	3	Nose	10-96	001110000000000	7168	XI-6	A	F_1	FFB	NR	+/+	9	+	−	+	−
VIId	F2	4	Nose	10-96	001110000000000	7168	XI-6	A	F_1	FFB	NR	+/+	9	+	−	+	−
VIIe	F2	5	Nose	10-96	001110000000000	7168	XI-6	A	F_1	FFB	NR	+/+	9	+	−	+	−
VIIIa	G	1	Sputum	01-96	100111110111101	20413	Z-72	A	G	GGF	D	−/−	10	+	−	+	−
VIIIb	G	2	Unknown	03-96	100111110111101	20413	Z-72	A	G	GGF	D	−/−	10	+	−	+	−
VIIIc	G	3	Unknown	04-96	100111110111101	20413	Z-72	A	G	GGF	D	−/−	10	+	−	+	−
VIIId	G	4	Pus	04-96	100111110111101	20413	Z-72	A	G	GGF	D	−/−	10	+	−	+	−
VIIIe	G	5	Nose	04-96	100111110111101	20413	Z-72	A	G	GGF	D	−/−	10	+	−	+	−

IXa	H	1	Urine	01-96	10111001010101101	23725	III-29	A	H	HAA	D	−/−	9	+	−	+	−
IXb	H	1	Urine	01-96	10111001010101101	23725	III-29	A	H	HAA	D	−/−	9	+	−	+	−
IXc	H	2	Pus	01-96	10111001010101101	23725	III-29	A	H	HAA	D	−/−	8	+	−	+	−
IXd	H	3	Pus	01-96	10111001010101101	23725	III-29	A	H	HAA	D	−/−	9	+	−	+	−
IXe	H	4	Pus	01-96	10111001010101101	23725	III-29	A	H	HAA	D	−/−	9	+	−	+	−
X1	I	1	Ven. line	01-93	00111111010101011	8107	III-29	A	I	IAA	D	−/−	10	+	−	+	−
X2	I	2	Nose	01-93	00111111010101011	8107	III-29	A	I	IAA	D	−/−	10	+	−	+	−
X3	I	3	Wound	02-93	00111111010101011	8107	III-29	A	I	IAA	D	−/−	10	+	−	+	−
X4	I	4	Bile	02-93	00111111010101011	8107	III-29	A	I	IAA	D	−/−	10	+	−	+	−
X5	I	5	Wound	02-93	00111111010101011	8107	III-29	A	I	IAA	D	−/−	10	+	−	+	−

[a] See Table 1, collection 10 for more details.
[b] Overall results after hybridization with 15 strain-specific DNA probes (AW-1 through AW-15, respectively).
[c] Binary code transformed into a decimal number.
[d] MSSA strains have identical phage types within a cluster. Types were dropped on account of the extensive code.
[e] Results with *Eco*RI-digested genomic DNA.
[f] After *Sma*I macrorestriction analysis.
[g] The three letter code summarizes the typing results per primer used (first letter, primer ERIC-2; second letter, primer AP-1; third letter, primer AP-7).
[h] RFLP analysis of the coagulase gene and subsequent digestion of the amplicon with *Alu*I. NR, no restriction sites on the amplicon.
[i] PCR result with *cna* gene-specific primers is presented before the shill, and the plus or minus after the shill describes the hybridization result with the PCR-generated, *cna*-specific DNA probe.
[j] Analysis of the so-called X region by PCR. Amplicons were digested with *Rsa*I. The number of direct repeats in this region was determined after electrophoresis.
[k] −, absence of the *mec*-specific DNA regions analyzed by PCR; +, presence of the *mec*-specific DNA regions analyzed by PCR.
[l] Patient removed from hospital B3 to an annex, hospital B1.
[m] Patient was transferred from nursing home D to hospital C.
[n] Outbreak in a hospital on the Netherlands Antilles.
[o] F1 and F2 are subdivisions in different locations of the same hospital.

sequential isolates recovered from healthy individuals who were shown to be persistent nasal carriers of *S. aureus* (43). The persistent carriers were monitored in 1988 and 1995, and similarities of the genotypes among these two sampling periods were determined with binary typing, PFGE, RAPD analysis, coagulase and protein A gene polymorphism, and the absence or presence of the *cna* gene. All genomic characterization techniques (Table 3), including the 15 epidemiological markers of the binary typing system, indicated a high degree of genomic stability over the years, except for the *spa* gene typing (persistent carrier 060). During laboratory storage and replication, mutations and transpositional recombination may occur (6), and the stability of the epidemiological markers for the staphylococcal genome can be measured by in vitro stability. The in vitro stability of the binary typing system was estimated by comparing the genomes of strains before and after 50 serial passages of strains on culture media. All DNA probes generated identical results after repeated testing (data not shown).

The Simpson index of diversity (*D*) expresses the discriminatory power of a genotyping system (17, 18). We calculated the *D* value for binary typing and compared this with the results of other frequently used techniques (Table 4, PFGE, RAPD analysis, *mecA*-Tn*554* probing). Certain systems, such as PFGE or RAPD analysis with multiple primers, generate complex banding patterns, and the Simpson index was calculated on the basis of the similarity level, defining a genotype (36). Hunter (18) proposed that the standardized discrimination index determines the discrimination index of a typing method that has a reproducibility of 95%; this is designated D_{95}. Both binary typing and PFGE exceed the level of D_{95}, and consequently these methods can be used as a single method. Less discriminating systems such as RAPD analysis and *mecA*-Tn*554* probing can be used in combination to obtain a significant D_{95} index (18, 33).

The probability of clonal linkage among epidemic strains determined to be similar by diverse genotyping techniques can be expressed at the level of reproducibility. In fact an application of in vivo stability, i.e., comparison of sequential isolates, recovered along the course of a well-documented outbreak (33). The whole-genome characterization techniques binary typing, PFGE, and RAPD analysis display adequate reproducibility among the related genomes of the epidemic MRSA strains (Table 5, clusters V through X). Only the number of repeats within the *spa* gene remain unstable within genetically related strains, and no concordance is demonstrated for analyzing presence versus absence of specific genes (*cna*, *mecA*, *mecI*, and *mecR1*). Strains originated from different locations of hospital F (Table 5, clusters VI and VII) are genetically related, as shown by the genotyping results.

Conclusion and future developments. The binary typing method described herein provides a reproducible, high-resolution molecular typing system strategy that may in the end be preferred over other means of genotyping. This method generates a simply binary output which is to be preferred over the complex banding patterns generated by most other genotyping systems. Furthermore, an important advantage of the binary typing system compared to other genotyping systems is that the system essentially comprises an assay procedure that is amenable to extensive automation and does not require variation in electrophoretic conditions such as voltage, time of run, and temperature, etc. (38, 42). Moreover, DNA hybridization can be performed by using an enzyme-linked immunosorbent assay-like technique, allowing implementation of this approach in most routine microbiological laboratories. It is theoretically also possible to develop specific DNA probes to determine virulence factors and resistance determinants for additional

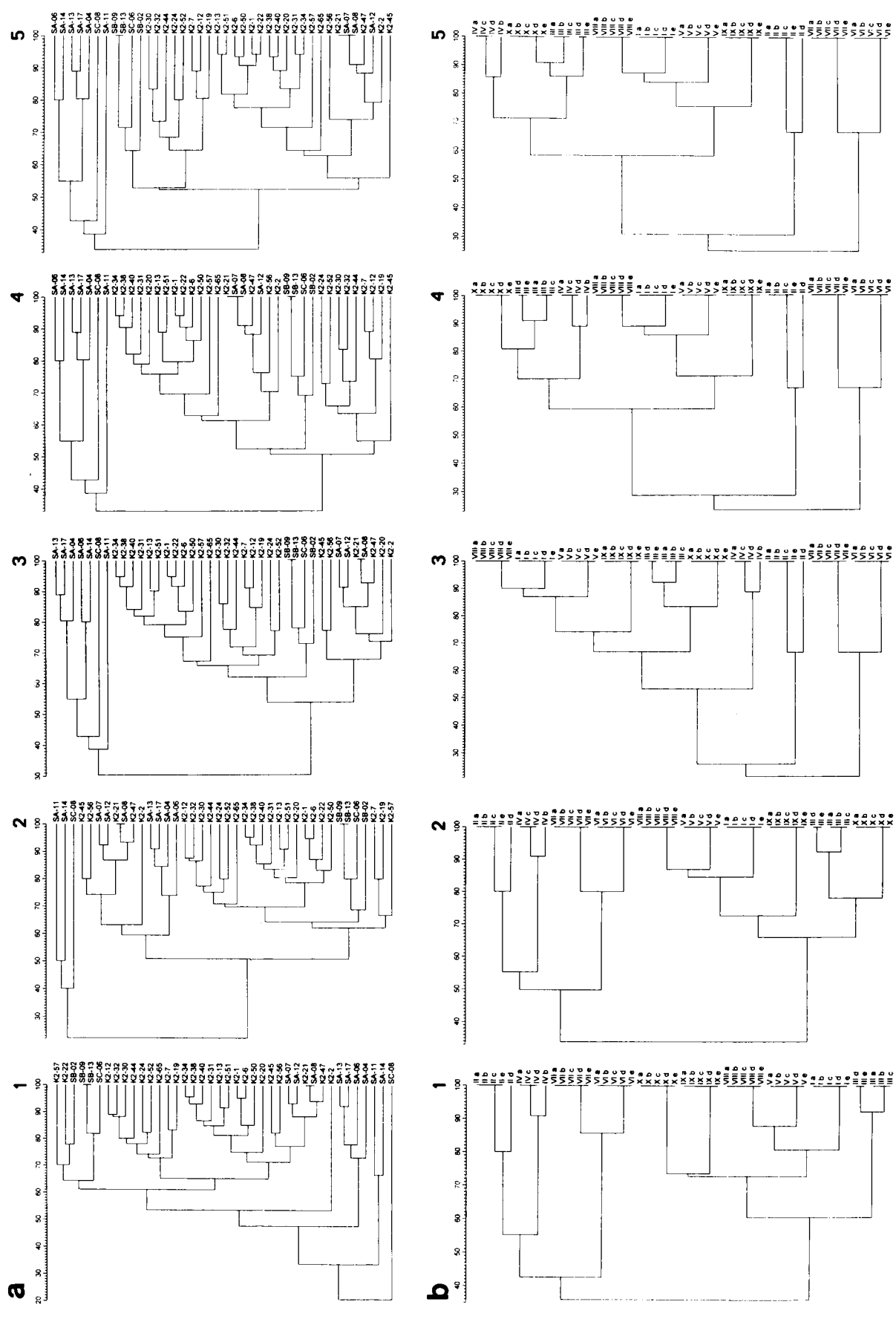


FIG. 3. (a) Dendrograms displaying the grouping of 40 unique *S. aureus* strains (Table 1, collections 6 and 7) on the basis of hybridization scores after binary typing with all DNA probes (dendrogram 1); deletion of probe AW-4 (dendrogram 2); deletion of probes AW-4 and AW-3 (dendrogram 3); deletion of probes AW-4, AW-3, and AW-15 (dendrogram 4); and deletion of probes AW-4, AW-3, AW-15, and AW-6 (dendrogram 5). (b) Dendrograms presenting the similarity percentages of the hybridization patterns of 10 outbreak clusters of *S. aureus* strains (Table 1, collection 10) obtained with the complete panel of DNA probes comprising the binary typing system (dendrogram 1); after deletion of probe AW-4 (dendrogram 2); after deletion of probes AW-4 and AW-3 (dendrogram 3); after deletion of probes AW-4, AW-3, and AW-15 (dendrogram 4); and after deletion of probes AW-4, AW-3, AW-15, and AW-6 (dendrogram 5).

diagnostic information (15). In principle, this technique can be extrapolated easily to other bacterial species (12). The binary typing system satisfies the requirements of the accepted performance criteria and promises to become a technically simple and fast library typing system.

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