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Typing of *Staphylococcus aureus* by Pulsed-Field Gel Electrophoresis, Zymotyping, Capsular Typing, and Phage Typing: Resolution of Clonal Relationships

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Sixty-nine *Staphylococcus aureus* isolates from two epidemiologically unrelated sources were typed by pulsed-field gel electrophoresis after *Sma*I digestion of chromosomal DNA (genome typing), and the results were compared with those obtained by other typing methods: phage typing with the international set of phages, capsular serotyping with monoclonal antibodies against capsular polysaccharides type 5 and 8, and zymotyping by polyacrylamide agarose electrophoresis for esterase polymorphism. A good correlation of *S. aureus* types was found by these four typing methods. Differentiation increased in the order capsular typing < zymotyping < phage typing < genome typing, yielding 2, 10, 20, and 26 different *S. aureus* types, respectively. Five of the 26 genome types were further divided into several subtypes revealing clonal relationships. When 36 French *S. aureus* isolates were compared with 33 German *S. aureus* isolates, 3 strains representing clonal populations were identical in both groups. *S. aureus* isolates from patients with cystic fibrosis were also typed at the beginning and the end of a 4-week summer camp for these patients. The results suggested a possible strain transmission during the summer camp. We conclude that genome typing by pulsed-field gel electrophoresis is a powerful tool not only for strain identification but also for the resolution of the clonal relationships of *S. aureus* strains.

Staphylococcus aureus is a major human pathogen that produces a wide spectrum of diseases. In recent years, strains of *S. aureus* resistant to multiple antibiotics have caused increasing problems of nosocomial infections in many parts of the world (3, 11, 19, 23, 24). An important strategy to reduce the number of *S. aureus* infections may be the interruption of strain transmission between susceptible patients or between healthy individuals (nasal carriers) and patients. Such epidemiologic studies depend on the availability of highly discriminatory typing systems which may differentiate strains from different sources.

A variety of systems have been used for *S. aureus* typing, such as phage typing (4), capsular polysaccharide serotyping (2, 5, 7, 15, 17, 32), protein analysis (10, 12, 18), zymotyping by analyzing alloenzyme patterns (6–8), ribotyping (13, 20, 28), and plasmid typing (3, 11, 18, 19). The success of the generally used phage typing can be limited by poor reproducibility of the results and the occurrence of *S. aureus* strains that are not typeable by this method (3, 24). Plasmid typing is limited to strains which bear plasmids, and strains may change or even loose their plasmid contents (18, 19). On the other hand, zymotyping has proved to be a stable and reproducible method of strain differentiation (6, 7). Additionally, DNA fingerprinting of chromosomal DNA by ribotyping or pulsed-field gel electrophoresis (genome typing) seems to be useful, since it was successfully used to type various bacteria (1, 9, 14, 20, 23, 26).

In the study described here we compared genome typing with established and commonly used typing methods such as phage typing, capsular serotyping, and zymotyping. Sixty-nine *S. aureus* isolates from two different epidemiologically unrelated sources were first classified according to the pulsed-field patterns of the strains. By correlating the results of the four typing methods, the definition between types and subtypes in genome typing appears reasonable.

MATERIALS AND METHODS

Bacterial strains. Thirty-six *S. aureus* isolates from patients attending six French hospitals were used in the different typing procedures. The isolates have been described elsewhere (15). Additionally, 33 *S. aureus* isolates were collected from 20 of 53 patients with cystic fibrosis (CF) attending a 4-week summer camp in Germany (Damshöhe) in 1990; collections were made at the beginning and at the end of the stay. These two sources of isolates were considered epidemiologically unrelated, since there was no contact between the French and the German groups of patients. All *S. aureus* isolates were identified by routine methods and were stored at −70°C.

Phage typing. The international basic set of 23 phages (group I, 29, 52, 52A, 79, and 80; group II, 3A, 3C, 55, and 71; group III, 6, 42E, 47, 53, 54, 75, 77, 83A, 84, and 85; group V, 94 and 96; and miscellaneous, 81 and 95) was applied by using standard methods (4). An additional experimental phage, A 994, was also tested. Isolates were typed both at the routine test dilution and at a 100-fold higher concentration. Only reactions showing major lysis were considered, as recommended previously (4).

Capsular typing. Type 5 and type 8 capsular polysaccharides were detected in bacteria grown on Columbia agar slants (Difco Laboratories, Detroit, Mich.). The bacteria were suspended in phosphate-buffered saline and autoclaved

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at 121°C for 1 h. After centrifugation for 10 min at 10,000 × *g*, the polysaccharides were detected in the supernatant by inhibition enzyme-linked immunosorbent assay (5) by using purified capsular antigen preparations (16, 17) and the corresponding monoclonal antibodies. Strains lacking both type 5 and type 8 capsular polysaccharides were designated nontypeable.

Zymotyping. The electrophoretic mobility patterns of esterases were investigated as described previously (6, 22), with the following modifications. Esterases from supernatants of lysostaphin-treated staphylococcal cultures were analyzed by electrophoresis with a 5% acrylamide and 0.8% agarose gel in a discontinuous Tris-glycine buffer (pH 8.7) at a constant value of 7 V cm^{-1} until the dye marker had run 11 cm. Esterases were stained in the gel by using α-naphthyl propionate as the substrate.

Genome typing. Chromosomal DNA was isolated as described by Prévost et al. (29), with modifications. The isolates were grown in 10 ml of 2× YT liquid medium (25) at 37°C until growth reached 0.1 absorbance units (600 nm). A total of 100 μl of 0.5 M EDTA (pH 8.0) was added, and the culture was cooled to 4°C. The bacteria were harvested by centrifugation (6,000 × *g*) for 15 min at 4°C, washed twice in 1 ml of ice-cold washing buffer [10 mM Tris-HCl, 10 mM EDTA, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 M NaCl (pH 7.5)], and resuspended in 100 μl of the same buffer. The bacteria were heated to 55°C and mixed with 100 μl of 2% low-melting-point agarose in TE buffer (pH 8.0) (25). The mixture was poured into the slots of a plastic mold (Bio-Rad) and cooled for 20 min at 4°C. The agarose plugs that were obtained were then transferred into sterile cups containing 1 ml of lysis buffer (6 mM Tris-HCl, 100 mM EDTA, 1 M NaCl, 0.5% [wt/vol] Brij 58, 0.2% [wt/vol] sodium deoxycholate, 0.5% [wt/vol] lauroyl sarcosine [pH 7.6], 100 μg of lysostaphin [Sigma] per ml, and 500 μg of lysozyme [Serva] per ml). Lysis was performed overnight with gentle shaking at 4°C. The lysis buffer was then substituted for 1 ml of proteolysis buffer (0.25 M EDTA, 20 mM EGTA, 1% [wt/vol] lauroyl sarcosine [pH 9.0], 500 μg of proteinase K [Sigma] per ml). Proteolysis was performed for 24 h at 55°C with gentle shaking, with a change of buffer after 12 h. Finally, the agarose plugs were washed five times with 10 ml of TE buffer (pH 8.0) at 4°C with gentle shaking. The digestion was performed by placing a 2-mm slice of each plug in 100 μl of restriction buffer containing 15 U of *Sma*I (GIBCO Bethesda Research Laboratories). After incubation overnight at 25°C, the reaction was stopped with TE buffer. The plugs were placed into the slots of a 1% agarose gel in 0.5× TBE buffer (31), and the slots were sealed with 1% agarose. Electrophoresis was performed by using the contour-clamped homogeneous electric field system (Bio-Rad). The conditions were 200 V for 24 h at 12°C, with pulse times ranging from 1 to 45 s. *Saccharomyces cerevisiae* genomes and bacteriophage lambda DNA concatamers (Bio-Rad) were used as size standards. The gels were stained with ethidium bromide and photographed.

Oxacillin resistance. Susceptibility to oxacillin was determined by the standard disk method on Mueller-Hinton agar plates. Inhibition of growth was interpreted by standard recommendations. The disks contained 5 μg of oxacillin.

RESULTS

Typeability, reproducibility, and discriminatory power of the four typing methods. The results of the four different typing methods for 69 *S. aureus* isolates are given in Tables 1 and 2. Electrophoresis of *Sma*I digests yielded well-resolved patterns of 12 to 16 fragments of 10 to 650 kb (Fig. 1). The fragment patterns were stable features of the isolates and of the DNA isolation procedure. When the isolates were repeatedly subcultured, their DNA restriction patterns remained unchanged. A genome type was arbitrarily defined as any fragment pattern which varied from another pattern with regard to the number and size of the DNA fragments. Types with smaller differences in the fragment patterns were regarded as subtypes belonging to the same major type. All

TABLE 1. Results of different typing methods of 36 *S. aureus* isolates from patients attending six French hospitals[a]

Hospital	Isolate no.	LT	Ox	CT	ZT	GT
C	F15	54	R	5	6	13
C	F32	77	R	5	6	15a
HD	F28	NT	R	5	6	15b
AB	F35	77	R	5	6	15c
T	F48	77	R	5	6	15d
BI	F12	47, 54, 75, 77, 84, 85, +	R	5	6	19
C	F24	6, 47, 54, 75, 77, 84, 54A, +	R	5	6	19
C	F16	47, 54, 75, 77, +	R	5	6	22
BI	F18	53, 54, 75, 77, 84, 85, 54A, +	R	5	6	26
T	F46	42E, 53, 54, 77, 84, 54A	R	5	6	27
SJ	F44	77	R	5	6	29
SJ	F38	NT	S	8	7	6
AB	F26	7, 53, 54, 75, 77, 83A, 84, 54A, +	S	5	9	14
AB	F27	95	S	8	10a	7
C	F41	6, 54, 75, 85	R	8	14	16
AB	F11	NT	S	8	14	18
C	F42	81	S	8	14	18
HD	F17	NT	S	8	14	20a
BI	F19	NT	S	8	14	20b
SJ	F39	29	R	8	14	20c
C	F40	NT	S	8	14	20d
HD	F20	28, 80, 75, 77, 95, +	S	8	14	21a
SJ	F21	95, +	S	8	14	21b
SJ	F22	NT	S	8	14	21c
C	F23	NT	S	8	14	21d
C	F31	NT	S	8	14	23
BI	F33	NT	S	8	14	24
BI	F13	84	R	8	14	25
T	F49	84	R	8	14	31
C	F30	94, 96	S	5	16a	1c
SJ	F36	94, 96	S	5	16a	1c
HD	F29	94, 96	S	5	16a	1d
HD	F37	94, 96	S	5	16a	1d
HD	F34	94, 96	S	5	16a	1h
T	F45	94, 96	S	5	16a	1i
C	F43	94, 96	S	5	16a	1j

[a] LT, phage type; Ox, oxacillin susceptibility (R, resistant; S, susceptible); CT, capsular type; ZT, zymotype; GT, genome type; NT, nontypeable.

TABLE 2. Results of different typing methods of 33 *S. aureus* isolates from 20 patients with CF attending a summer camp in Germany[a]

Patient no.	Result at the following collection time:							
	Beginning of stay				End of stay			
	LT	CT	ZT	GT	LT	CT	ZT	GT
1	—	—	—	—	NT	8	7	6
2	94, 96	5	16a	1a	94, 96	5	16a	1c
	94, 96	5	16a	1b				
3	—	—	—	—	NT	8	23	3a
4	94, 96	5	16a	1c	—	—	—	—
5	NT	NT	5	2	—	—	—	—
6	94, 96	5	16a	1d	—	—	—	—
7	94, 96	5	16a	1d	94, 96	5	16a	1d
	NT	8	23	3a				
8	—	—	—	—	NT	NT	23	3b
9	—	—	—	—	NT	8	33	10
10	—	—	—	—	94, 96	5	16a	1d
11	94, 96	5	16a	1e	—			
	94, 96	5	16a	1f				
12	—	—	—	—	NT	8	23	3a
13	94, 96	5	16a	1c	94, 96	5	16a	1d
					94, 96	5	16a	1d
14	3C, 55, 71, 3C, 55, 71		8	2	4	—	—	—
			8	2	4			
15	52, +	8		5	—	—	—	—
16	55, +	8	7	6	94, 96	5	16a	1c
17	95	8	10a	7	—	—	—	—
18	NT	8	7	6	NT	8	23	3a
	95	8	10a	7				
19	—	—	—	—	NT	8	23	3a
					NT	8	23	3a
20	6, 47, 53, 54, 75, 83A, 85, A994, +	NT	5	8	29	NT	14	11
					A994			

[a] LT, phage type; CT, capsular type; ZT, zymotype; GT, genome type; —, no isolates obtained; NT, nontypeable.

isolates were typeable (100%) by this method and were differentiated into 26 genome types. The most frequent type was G1. This type was further divided into 10 additional subtypes (G1a to G1j). Subtypes could also be distinguished in four other types (G3a and G3b, G15a to G15d, G20a to G20d, and G21a to G21d).

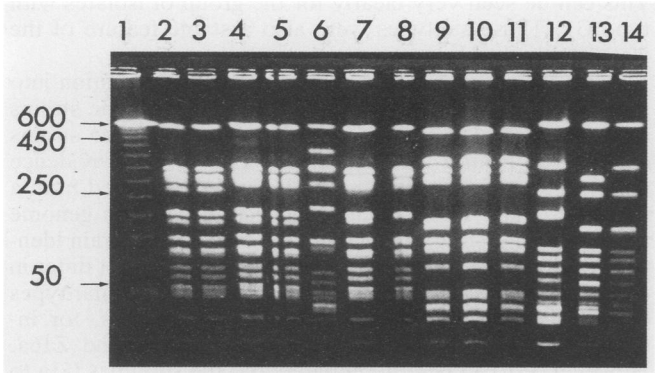

FIG. 1. Pulsed-field gel electrophoresis of *Sma*I-digested chromosomal DNA from *S. aureus*. For experimental details, see text. Molecular masses (in kilobases) were obtained from bacteriophage lambda DNA concatemers (lane 1). Lanes 2 to 5, 7, and 8, isolates 3, 19, 33, F29, 8, and 9 (genome type 1), respectively; lane 6, isolate F24 (genome type 19); lanes 9 to 11, isolates 7, 27, and 29 (genome type 3), respectively; lanes 12 to 14, isolates F39, F40, and F17 (genome type 20), respectively.

By phage typing, 20 different phage types were identified; by capsular typing, only two capsular types were identified. The isolates were differentiated into 10 different zymotypes by esterase polymorphism. The typeability of the isolates by zymotyping was 100%.

Differentiation increased in the order capsular serotyping < zymotyping < phage typing < genome typing, yielding 2, 10, 20, and 26 different *S. aureus* types, respectively. Sixteen isolates (23%) were found to be resistant to oxacillin.

Correlation of the four typing methods. To investigate further whether the results obtained by the four different typing methods correlated with each other, the following questions were asked: (i) How often are two isolates with identical genome types different in phage type, capsular type, and zymotype? (ii) How often are two isolates with different genome types identical in phage type, capsular type, and zymotype? (iii) How often are two isolates with identical genome types identical by the other typing methods? The results reveal that isolates with identical genome types never differ in capsular type or zymotype. The phage type differed twice, although the genome type was identical. Often, isolates with identical capsular types and zymotypes belonged to different genome types. This is in line with the higher discriminatory power of genome typing. Often, genome type, capsular type, and zymotype were found to be identical. Figure 2 shows the relation between the best

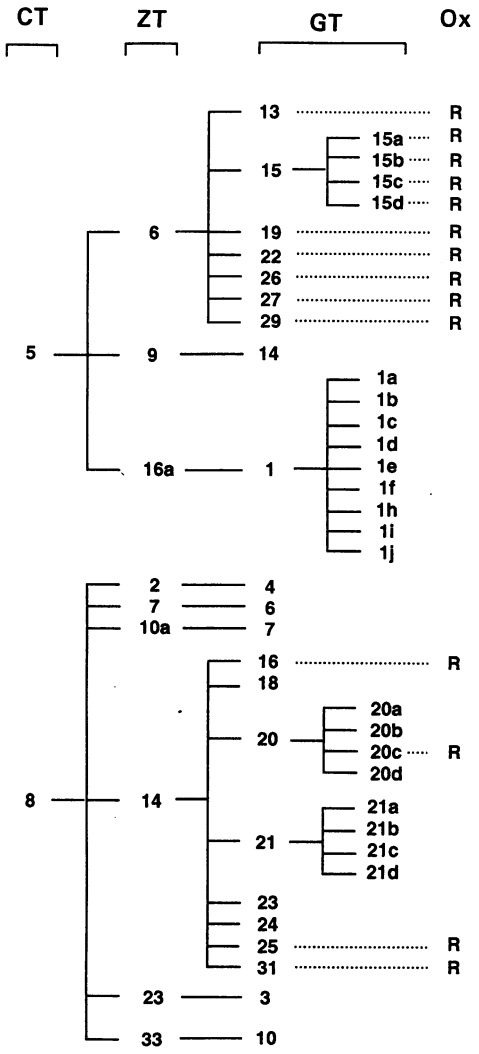

FIG. 2. Clonal relationships established with capsular type (CT), zymotype (ZT), and genome type (GT) of *S. aureus* isolates. Oxacillin (Ox)-resistant isolates are indicated with an R. The two capsular types 5 and 8 could be differentiated into 9 zymotypes and 22 genome types.

correlating markers capsular type, zymotype, and genome type.

Comparison of French and German *S. aureus* isolates. We examined whether isolates from independent patient groups show similar *S. aureus* types. Therefore, the genome types of the 36 isolates from six French hospitals were compared with 33 isolates from patients with CF obtained during a summer camp in Germany (Tables 1 and 2). Three genome types (G1, G6, and G7) were present in both groups. The most common type was the oxacillin-susceptible strain G1, G6 and G7 were rare. Similar results were obtained by the other typing methods. Thus, the appearance of identical genome types does not necessarily indicate an epidemic situation.

Transmission of *S. aureus* during a summer camp for patients with CF? The four typing methods were used to investigate the distribution of *S. aureus* strains at the beginning and the end of a 4-week summer camp for patients with CF. Thirty-three *S. aureus* isolates were collected from 20 of the 53 patients with CF, 18 isolates at the beginning and 15 isolates at the end of the stay (Table 2). Genome typing revealed that G3a isolated from patient 7 was present in four other patients (patients 3, 12, 18, and 19) at the end of the stay. Whether such findings are significant for an epidemic spread of G3a during the summer camp by cross-colonization, however, remains to be clarified by larger studies.

DISCUSSION

The importance of typing *S. aureus* strains or strains of other bacterial species for epidemiological reasons is generally acknowledged; however, the choice of the right typing method is often difficult because of inherent theoretical and technical limitations. Thus, for example, the generally used phage typing is often unsatisfactory in differentiating *S. aureus* strains, since its typeability is less than those of other typing methods (3, 24). In the present study, 30% of the isolates were nontypeable by this method, whereas all isolates were typeable by zymotyping or genome typing. On the other hand, the degree of discrimination of phage typing is normally high, which is in contrast to its questionable reproducibility, at least in the hands of some investigators (3). Capsular typing has only a very low power of discriminating between *S. aureus* strains; however, the validity of this method is confirmed by zymotyping and genome typing since it allows distribution of zymotypes and genome types into two populations (Fig. 2). Thus, a major advance in this context was by no means the introduction of zymotyping and genome typing, as also shown in the present study of 69 *S. aureus* isolates. However, since the discriminatory powers of capsular typing, zymotyping, and probably also phage typing are restricted, individual *S. aureus* strains or clonal populations are not detectable by these methods. Therefore, we propose that the expression "strain" is valid only for genome types. By using genome typing, it was possible to discriminate 26 different *S. aureus* strains. The genome types represent stable differences in the investigated *S. aureus* strains, since DNA fragment patterns remained unchanged even after numerous DNA preparations and subculturing of the isolates. Similar findings have been reported by Prévost et al. (29) and Goering and Duensing (20). In five cases the types could be divided into further subtypes according to minute differences in the fragment patterns. This can be seen very clearly for the group of isolates with type G1. These subtypes were also a stable feature of the isolates.

Here, the question arises whether this differentiation into subtypes represents true strain differences, i.e., the strains are indeed unrelated, or strain relatedness, i.e., the strains are derived from a common clone. Two lines of evidence allow us to conclude that the subtypes were derived from a common clone. First, a good correlation between genome typing and the other typing methods concerning strain identity was found, since identical genome types did not differ in capsular type or zymotype, whereas identical capsular types or zymotypes may differ in genome types. Thus, for instance, all G1 types belonged to L94,96, C5, and Z16a. However, G1 can be differentiated into the subtypes G1a to G1j. Similarly, Prévost et al. (28) tested the correlation between ribotyping and genome typing. Again, ribotypes could be differentiated further by genome typing, but genome types could not be differentiated further by ribotyping.

Second, the hypothesis that genome typing detects clonal variants of one strain is supported by the analysis of other microorganisms. The higher discriminatory power of ge-

nome typing over multilocus enzyme electrophoresis in *Escherichia coli* was discussed by Arbeit et al. (1) as evidence for the recent evolutionary divergence of related lineages. Miranda et al. (26) and Römling et al. (30) drew similar conclusions from their studies of *Enterococcus faecium* and *Pseudomonas aeruginosa*, respectively. Minor chromosomal variations may occur over time in one patient, as was shown for *Staphylococcus epidermidis* by Goering and Winters (21). This is in accordance with the results of our study, in which we observed that a single patient can be colonized with various subtypes of a given genome type. Therefore, genome typing is a tool that can be used to track the genetic development of a given *S. aureus* strain. However, more work (e.g., computer analysis) must be done to define strain relatedness and unrelatedness more accurately. Recently, El-Adhami et al. (14) estimated DNA similarities among methicillin-resistant *S. aureus* isolates from a single outbreak using numerical analysis. We think that investigators must be cautious in defining isolates with only minor changes in the pulsed-field gel electrophoresis pattern as different strains rather than as subtypes of one strain. This may lead to misleading conclusions in epidemiological studies because possible transmissions of strains will be undetected.

A further difficulty in the epidemiology of *S. aureus*, particularly in analyzing routes of transmission, is the present finding that in groups of patients without any possible contact, identical *S. aureus* genome types were detected. Thus, for example, G1, G6, and G7 were found among patients from six French hospitals and among patients with CF attending a summer camp in Germany. Therefore, G1, G6, and G7 seem to be ubiquitous strains. Witte (34) noticed an increase in the occurrence of phage type 94/96 in the former German Democratic Republic from 1979 to 1985. These results are especially remarkable compared with results of similar studies with other bacteria, e.g., *P. aeruginosa*. It was shown that patients with identical types usually had contact with a common source (33). The occurrence of identical strains in diverse geographic regions seems to be a specific feature of *S. aureus*. In this context it was discussed, for example, that some of the methicillin-resistant *S. aureus* strains are probably derived from a common clone (27). Whether this is due to the fast spread of some specific strains, as suggested by Bygraves and Maiden (9) for *Neisseria meningitidis*, or to a slow genetic development of *S. aureus* needs to be clarified.

The difficulties with epidemiological studies of *S. aureus* mentioned above also make it difficult to decide whether strain transmission occurred during the 4-week summer camp for patients with CF, where *S. aureus* type G3a, isolated from one patient at the beginning of the stay, was isolated from four patients at the end of the stay.

We conclude that DNA fingerprinting by pulsed-field gel electrophoresis is a powerful tool not only for strain identification but also for follow-up of the genetic development of natural *S. aureus* populations.

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