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Molecular Typing of *Staphylococcus aureus* on the Basis of Coagulase Gene Polymorphisms

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Staphylocoagulase, a major phenotypic determinant of *Staphylococcus aureus*, exists in multiple allelic forms, in part because of the existence of gene variants within the 3′-end coding region. This region contains a series of repeating 81-bp DNA sequences which differ both in the number of tandem repeats and the location of *Alu*I restriction sites among different isolates. Utilizing this finding, we developed a novel typing method for *S. aureus* based on polymerase chain reaction amplification of the variable region of the coagulase gene followed by *Alu*I restriction enzyme digestion and analysis of restriction fragment length polymorphism (RFLP). Among 30 *S. aureus* isolates studied initially, a total of 10 distinct RFLP patterns were observed. There was excellent correlation of the RFLP patterns with typing of these isolates by multilocus enzyme electrophoresis at 20 chromosomal loci. This coagulase RFLP method was used to analyze an additional 39 *S. aureus* isolates and successfully traced the source of an outbreak of methicillin-resistant *S. aureus* infections at a local hospital.

Coagulase production is the principal criterion used by the clinical microbiology laboratory for the identification of *Staphylococcus aureus* isolates from human infections. Numerous allelic forms of *S. aureus* coagulase exist, with each isolate producing one or more of these enzyme variants (3, 4, 6, 15). Analysis of the DNA sequences of cloned coagulase genes from three different *S. aureus* strains revealed that variable sequences exist within the 3′ coding regions of the allelic genes (7, 8, 14). On the basis of these observations, we surmised that different *S. aureus* isolates could be differentiated by virtue of these unique nucleotide sequences of the coagulase gene. In this report, we describe such a system based on the amplification and subsequent restriction enzyme digestion of the polymerase chain reaction (PCR) product(s) of the 3′ end of the coagulase gene. This region contains a series of 81-bp tandem repeats, the number of which differs between strains (Fig. 1). Although the sequences of these repeats are well conserved, individual repeats differ in the presence or absence of an *Alu*I restriction site (7, 8, 14). By *Alu*I restriction enzyme digestion of the amplified gene product, it is possible to discriminate between *S. aureus* isolates by the restriction fragment length polymorphism (RFLP) of the 3′-end region of the coagulase gene.

MATERIALS AND METHODS

Bacterial strains. Thirty *S. aureus* isolates, collected from various clinical sources and previously characterized by multilocus enzyme electrophoresis (MLEE) at 20 chromosomal loci by the method of Musser et al. (13), were studied initially. An additional 39 *S. aureus* isolates (24 methicillin resistant and 15 methicillin sensitive) were obtained during the investigation of a nosocomial outbreak of methicillin-resistant *S. aureus* (MRSA) infections at a local hospital and were similarly studied.

Bacterial cell lysis. Each isolate was subcultured overnight in 1 ml of trypticase soy broth and washed by centrifugation (1,000 × *g*) in 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM disodium EDTA (Sigma, St. Louis, Mo.) (TE). The cell pellet was resuspended in 500 μl of TE containing 15 U of lysostaphin (Sigma) per ml and was incubated at 37°C for 1 h. To this was added 1 ml of lysing buffer (composed of 0.45% Nonidet P-40 [Sigma], 0.45% Tween 20, and 0.6 μg of proteinase K [Sigma] per ml in PCR buffer [50 mM KCl–1.5 mM $MgCl_2$–1 mg of gelatin per ml–10 mM Tris-HCl, pH 8.3]). The sample was further incubated for 1 h at 56°C. Proteinase K was inactivated by being heated at 95°C for 10 min.

PCR amplification. The 3′-end region of the coagulase gene was amplified by using the nested-primer technique (9). The outer primers were COAG1-ATACTCAACCGACGAC ACCG and COAG4-GATTTTGGATGAAGCGGATT, which hybridize to the coagulase gene at sites 1362 to 1381 and 2859 to 2878, respectively (*S. aureus* BB) (8). Cell lysate (10 μl) was added to a PCR mixture containing 1 μM (each) primer, 4 μl of 10-fold concentrate of PCR buffer, 200 μM each deoxynucleoside triphosphate (dNTP), and 1 U of *Taq* polymerase (BRL/GIBCO, Burlington, Ontario, Canada) in a final volume of 40 μl of deionized water. Each sample was subjected to 40 PCR cycles, each consisting of 30 s at 95°C, 2 min at 55°C, and 4 min at 72°C. For nesting, 1 μl of the first PCR reaction mixture was added to a fresh PCR reaction mixture containing 4 μl of 10-fold concentrate of PCR buffer, 200 μM each dNTP, 1 μM (each) nested primers COAG2-CGAGACCAAGATTCAACAAG and COAG3-AAAGAAA ACCACTCACATCA (which hybridize to sites 1632 to 1651 and 2589 to 2608, respectively) (8), and 1 U of *Taq* polymerase in a total volume of 40 μl of deionized water. This mixture was similarly subjected to 40 cycles of PCR amplification as described above.

Restriction enzyme digestion. Ten microliters of the nested PCR product was digested overnight with 10 U of the restriction endonuclease *Alu*I (Bethesda Research Laboratories, Inc.) according to the manufacturer's recommended protocol. Both the PCR products and the restriction digest fragments were detected by electrophoresis through a 4% Nusieve GTG agarose gel (FMC Bioproducts, Rockland,

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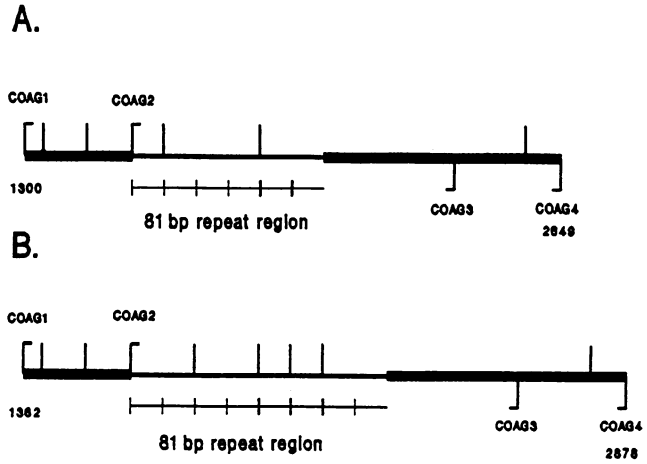

FIG. 1. Comparison of the locations of *Alu*I restriction sites within the coagulase genome of *S. aureus* strains 213 (A) and BB (B). Horizontal bars, primer binding sites utilized for constructing nested primers (COAG1, COAG2, COAG3, and COAG4) for PCR amplification; vertical bars, *Alu*I restriction enzyme recognition sites; vertical lines, areas containing variable 81-bp tandem repeat sequences.

Maine) in the presence of ethidium bromide and photographed under UV illumination.

RESULTS

The 30 isolates of *S. aureus* could be differentiated from each other on the basis of three characteristics of their PCR products, i.e., the presence of one or two PCR products, their size(s), and the *Alu*I restriction digest patterns of the PCR products. A single PCR product from 8 isolates was amplified, while two products from 22 isolates were amplified (Table 1). The sizes of the PCR products ranged from approximately 440 to approximately 915 bp (Fig. 2). Two isolates of coagulase-negative staphylococci (*S. epidermidis* and *S. haemolyticus*), which served as negative controls, produced no DNA products following PCR amplification (Fig. 2).

*Alu*I restriction enzyme digestion of the total nested PCR products generated multiple DNA fragments. Most isolates had a fragment of approximately 400 bp and bands in multiples of 81 bp (Fig. 3; Table 1). The presence or absence of these fragments allowed easy discrimination between different *S. aureus* isolates because of their unique coagulase gene RFLP patterns (Fig. 3). Furthermore, there was complete concordance between the results of typing by coagulase gene RFLP and those of MLEE among the 30 isolates tested (Table 1). A total of 10 distinct RFLP profiles corresponding to eight electrophoretic types (ETs) were recognized among these isolates. Interestingly, 14 isolates with

TABLE 1. Comparison of MLEE typing of *S. aureus* with molecular typing based on coagulase gene DNA fragment length polymorphism

ET	No. of isolates	PCR product (approx bp)	Presence of *Alu*I restriction digest fragment of the following size (bp)[a]:					
			81	162	243	324	405	486
1	4	480–730	+	+	−	−	+	−
2	1	730	−	−	−	+	+	−
3	2	730–980	+	−	+	−	+	+
4	1	710–810	−	−	+	+	−	+
5	9	355–610	+	+	−	−	+	−
5	4	460–915	+	−	−	+	+	−
5	1	500	−	−	−	−	−	+
6	2	390–810	−	+	−	−	+	+
7	4	760	−	+	+	−	+	−
8	2	610	−	−	−	−	+	+

[a] Results for only fragments of 81 bp or multiples are shown.

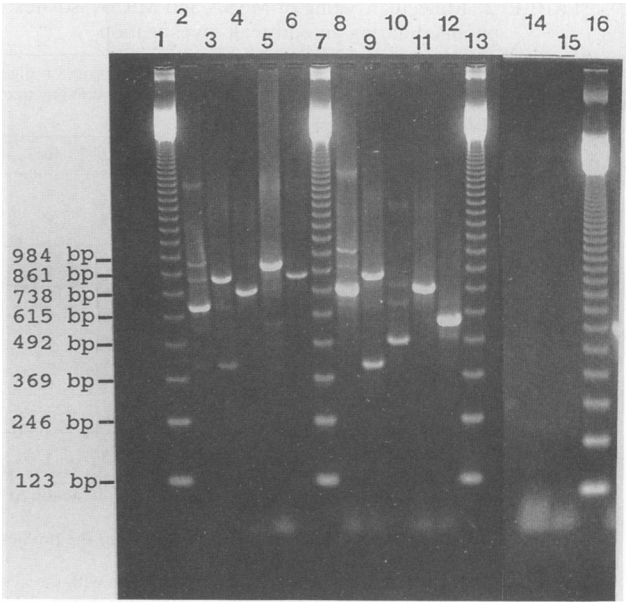

FIG. 2. Examples of the nested PCR coagulase gene products from different *S. aureus* isolates (lanes 2 to 6 and 8 to 12). Coagulase-negative staphylococci (lane 14, *S. epidermidis*; lane 15, *S. haemolyticus*) produced no coagulase gene products. Lanes 1, 7, 13, and 16 contain 123-bp molecular weight markers.

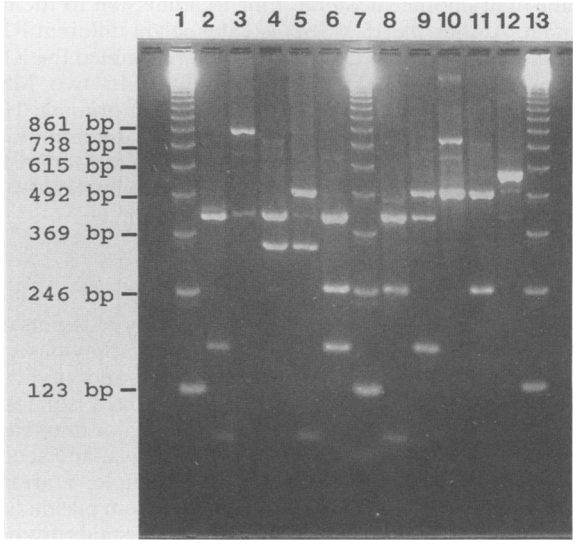

FIG. 3. *Alu*I restriction enzyme digest of the PCR coagulase gene products shown in Fig. 2. Lanes 1, 7, and 13 contain 123-bp molecular weight markers.

TABLE 2. Molecular typing of MSSA and MRSA isolates obtained during an outbreak investigation

MRSA	No. of isolates	PCR product (approx bp)	Presence of *Alu*I restriction digest fragment of the following size (bp)[a]:					
			81	162	243	324	405	486
+	23 (P11)[b]	490–710	+	+	+	−	+	−
+	1 (P9)[c]	860	−	+	−	+	−	+
−	2	450–800	−	−	−	−	+	−
−	1	700	−	−	−	−	+	−
−	2	830	+	−	+	−	+	−
−	5	420–650	+	−	−	−	+	−
−	2[d]	480–730	+	+	−	−	+	−
−	1	830	+	−	+	+	−	+
−	1	940[e]	−	+	−	+	−	+
−	1	940[e]	−	−	−	−	−	−

[a] Results for only fragments of 81 bp or multiples are shown.
[b] Isolate P11 was the index strain of the MRSA outbreak.
[c] Isolate P9 was cultured from a patient who had previously acquired the organism at another hospital.
[d] These two MSSA isolates have RFLP profiles identical to the profiles of the four isolates designated ET-1 in Table 1.
[e] These two MSSA isolates produced false-negative results with the nested primers COAG1, COAG2, COAG3, and COAG4. However, when COAG2 was substituted with a different primer, COAG2.1 (see text), a PCR product of approximately 940 bp was elaborated by these isolates.

the same ET designation (ET-5; Table 1) could be further differentiated into three subgroups by their distinct coagulase gene RFLP patterns, suggesting that typing by coagulase gene RFLP was more discriminating than typing by MLEE for these isolates.

To assess the clinical utility of our typing method, we carried out an analysis of 39 additional *S. aureus* isolates (24 MRSA and 15 methicillin-susceptible *S. aureus* [MSSA] isolates) collected from different patients during the investigation of a nosocomial outbreak of MRSA infections at a local hospital (Table 2). Of the 24 MRSA isolates, 23 were of the same RFLP type and all were epidemiologically linked to the index strain of the outbreak (isolate P11). The single MRSA isolate (P9) not showing the same RFLP pattern as the others was isolated from a patient who had acquired the organism at another hospital prior to admission to the local hospital. Among the 15 MSSA isolates, eight different RFLP patterns were identified, none of which resembled the RFLP profile of the MRSA isolates (Table 2). In two MSSA isolates, no PCR product was amplified after priming. However, by replacing the COAG2 primer with a different primer (COAG2.1) which hybridized to the sequence from bp 1456 to 1473 (8), a nested PCR product of approximately 940 bp was elaborated from these isolates (Table 2).

DISCUSSION

The method which we have developed to type strains of *S. aureus* has several advantages over those previously reported. Bacteriophage typing is laborious and of limited utility, since a significant percentage of *S. aureus* isolates are untypeable (18). Plasmid analysis has two major drawbacks: first, some isolates do not harbor any plasmids, and second, plasmids are inherently unstable. For example, rearrangements and/or deletions of DNA sequences from plasmids are common events, as is the loss of whole plasmids from the bacterium. For these very reasons, antibiogram profiles are not stable traits and cannot be used reliably for epidemiologic typing of *S. aureus* isolates. MLEE has been shown to be a powerful tool for population genetic studies of various bacteria, including *S. aureus* (12, 13), but this method is relatively labor-intensive.

Several investigators have described molecular typing of bacterial isolates on the basis of variations in the chromosomal DNA structure (1, 2, 5, 11, 16–18). Restriction digestion of total chromosomal DNA followed by pulsed-field gel electrophoresis can be used for typing of *S. aureus* (5), but because of the large number of fragments produced, these patterns may be difficult to interpret. Also, this method requires a large amount of purified intact chromosomal DNA. The coagulase gene RFLP method we describe here for typing of *S. aureus* isolates is far simpler than those previously reported. It requires only small quantities of crude DNA, and individual strains can be compared easily by both the number of PCR-amplified gene products and the sizes of their *Alu*I restriction enzyme digest fragments.

The 69 *S. aureus* isolates in the present study can be classified into 19 distinct groups based on their PCR-amplified coagulase gene products and unique *Alu*I RFLP profiles (Tables 1 and 2). This would constitute a minimum number of coagulase gene variants because of the limited number and selection of bacterial isolates used for this preliminary investigation. False-negative results for two isolates (3%) could be eliminated by improvements in selecting more appropriate alternate nested-primer sites (such as COAG2.1) for COAG2 (which might be too close to the variable region of the coagulase gene). Our data, compared with data from the MLEE typing method, strongly suggest that the coagulase gene RFLP typing method for *S. aureus* may be even more discriminatory than ET analysis. For example, isolates designated ET-5 by MLEE in this study can be further categorized into three distinct RFLP profiles on the basis of their coagulase gene polymorphism (Table 1).

To test the potential utility of the coagulase gene typing method, we analyzed 39 *S. aureus* isolates recovered from different patients and hospital personnel during the investigation of an outbreak of MRSA infections at a local hospital. Of 24 MRSA isolates, 23 were epidemiologically linked to the outbreak and all had identical coagulase gene RFLP profiles (Table 2). The single MRSA isolate (P9) with a different RFLP profile was isolated from a patient who was known to be an MRSA carrier prior to hospital admission. Isolates P9 and P11 were the first MRSA isolates recovered from this community hospital. Our typing method demonstrated that isolate P11, not isolate P9, was the index strain which accounted for the MRSA outbreak. All 15 MSSA isolates recovered from patients and hospital personnel during the outbreak investigation could be categorized into eight unique RFLP profiles, none of which resembled those of the MRSA isolates. This preliminary study clearly demonstrates the potential usefulness of this coagulase gene polymorphism method for the investigation of nosocomial outbreaks due to either MRSA or MSSA infections.

At least eight serologically distinct coagulase enzymes have been identified in different *S. aureus* isolates (6). In some instances, up to four immunologically distinct forms of the enzyme were produced in the same isolate (3, 15). Since our primers amplified across a variable portion of the coagulase gene and since some isolates gave rise to more than one amplified PCR product, our data strongly suggest that these same *S. aureus* isolates have more than one allelic form of the coagulase gene. It is interesting that *S. aureus* BB has eight tandem repeat sequences of 81 bp each at the 3′-end coding region of the coagulase gene (8). Analysis of the nucleotide sequence data from *S. aureus* strains 213 (7)

and 8325-4 (14) showed that they have only six and five tandem 81-bp repeat sequences, respectively. This results in the encoding of coagulases with molecular weights different from that of the coagulase of *S. aureus* BB. Analysis of the DNA repeat regions of strains BB and 213 further revealed differences in *Alu*I restriction enzyme recognition sites (Fig. 1). We believe that such DNA sequence differences in the staphylocoagulase gene are responsible for the polymorphic restriction fragment length patterns seen among the *S. aureus* isolates we have studied. The teleologic reason for this polymorphism in the coagulase gene among *S. aureus* strains is unclear, but the extensive polymorphism observed does suggest that the coagulase gene may be an important virulence determinant for this organism. However, it should be noted that the carboxyl end of the coagulase encoded by this repetitive region does not appear to be required for its prothrombin-activating function (10).

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