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Capsule Expression and Genotypic Differences among *Staphylococcus aureus* Isolates from Patients with Chronic or Acute Osteomyelitis[▽]

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There is ample evidence that *Staphylococcus aureus* capsular polysaccharide (CP) promotes virulence. Loss of capsule expression, however, may lead to *S. aureus* persistence in a chronically infected host. This study was conducted to determine the relative prevalence of nonencapsulated *S. aureus* in patients with chronic and acute osteomyelitis. Only 76/118 (64%) *S. aureus* isolates from patients with osteomyelitis expressed CP, whereas all 50 isolates from blood cultures of patients with infections other than osteoarticular infections expressed CP ($P = 0.0001$). A significantly higher prevalence of nonencapsulated *S. aureus* was found in patients with chronic osteomyelitis (53%) than in those with acute osteomyelitis (21%) ($P = 0.0046$). *S. aureus* isolates obtained from multiple specimens from five of six patients with chronic osteomyelitis exhibited phenotypic (expression of CP, α-hemolysin, β-hemolysin, slime, and the small-colony variant phenotype) and/or genotypic (pulsed-field gel electrophoresis and *spa* typing) differences. Nonencapsulated *S. aureus* was recovered from at least one specimen from each chronic osteomyelitis patient. Fourteen isolates obtained from two patients with acute osteomyelitis were indistinguishable from each other within each group, and all produced CP5. In conclusion, we demonstrated that nonencapsulated *S. aureus* is more frequently isolated from patients with chronic osteomyelitis than from those with acute osteomyelitis, suggesting that loss of CP expression may be advantageous to *S. aureus* during chronic infection. Our findings on multiple *S. aureus* isolates from individual patients allow us to suggest that selection of nonencapsulated *S. aureus* is likely to have occurred in the patient during long-term bone infection.

The development of prosthetic joints has been one of the biomedical success stories of the 20th century. However, despite improvements in surgical techniques, implantation of medical devices is associated with a definite risk of bacterial infection. Among the different etiological agents causing prosthetic device-associated infections, *Staphylococcus aureus* emerges as one of the most prevalent and difficult-to-eradicate pathogens (25). This pathogen has acquired resistance to virtually all of the antimicrobial agents available, and in recent years, the worldwide emergence of multiresistant clones in hospitals and communities has spurred significant concern (32). *S. aureus* pathogenicity is due to a vast array of virulence factors, many of which are of broad functionality with considerable redundancy, allowing staphylococci to readily adapt to various environmental niches. There is still no clear knowledge, however, about the individual factors associated with the chronicity of *S. aureus* infections. Experimental studies conducted in our laboratory with a mouse model of staphylococcal mastitis demonstrated that lack of capsular polysaccharide (CP) expression results in an increased capacity for persistence in the host (35). This capacity was attributed to more effective interaction between unmasked staphylococcal surface ligands and cell receptors, leading to internalization into epithelial cells (8, 35). Studies performed with clinical *S. aureus* isolates from bovines with subclinical mastitis revealed a high prevalence of strains that express neither CP serotype 5 (CP5) nor CP8 (33). The potential medical importance of *S. aureus* strains that do not express CP5 or CP8 (nontypeable, NT) has been underscored (10). The major mechanisms responsible for lack of *S. aureus* CP expression have been thoroughly described and include point mutations in the *cap5*(*8*) promoter or in 1 of the 11 genes essential for capsule production (10). Other strains have mutations in genes that regulate capsule expression, such as *agr* or *arlRS* (10). In one particular region, many NT strains of *S. aureus* from bovine mastitis fail to produce CP because the *cap5*(*8*) locus suffered an IS*cap*-mediated deletion (36). We hypothesize that loss of CP5 (CP8) expression provides *S. aureus* a selective advantage for persistence in certain infected hosts. If this is so, other hosts with chronic *S. aureus* infections would also exhibit a high prevalence of NT variants.

The present study was conducted to determine the relative prevalence of NT *S. aureus* in 118 patients with chronic versus acute osteomyelitis. In addition, we investigated multiple *S.*

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TABLE 1. Encapsulated and NT *S. aureus* isolates recovered from patients with osteomyelitis or isolated from the blood of patients with a focus of infection other than osteoarticular infection

Infection	No. (%) of encapsulated isolates, no. $CP5^+$/no. $CP8^+$[a]	No. (%) NT	Total no.	*P* value[b]
Osteoarticular	76 (64), 57/19[c]	42 (36)	118	
Other (blood culture)	50 (100), 33/17[c]	0 (0)	50	0.0001

[a] The values in parentheses represent isolates that express CP as ascertained by colony immunoblotting and immunodiffusion.
[b] Statistical significance of difference, Fisher exact test.
[c] The difference in the proportion of $CP5^+/CP8^+$ *S. aureus* isolates between the two groups (osteoarticular infection versus other) was not statistically significant.

TABLE 2. Encapsulated and NT *S. aureus* isolates from patients with chronic or acute osteomyelitis[a]

Osteomyelitis	No. (%) of encapsulated isolates[b]	No. (%) of NT isolates	Total no.	*P* value[c]
Chronic	31 CP^+ (47)	35 (53)	66	0.0046
Acute	26 CP^+ (79)	7 (21)	33	
Chronic	25 $CP5^+$ (54)	21 (46)[d]	46	0.027
Acute	16 $CP5^+$ (84)	3 (16)[d]	19	
Chronic	6 $CP8^+$ (30)	14 (70)[e]	20	0.035
Acute	10 $CP8^+$ (71)	4 (29)[e]	14	

[a] The proportion of capsulated (CP^+) and NT *S. aureus* isolates between groups of patients with chronic and acute disease was also compared within each CP serotype. A total of 99 patients were included.
[b] The values represent isolates that express CP as ascertained by colony immunoblotting and immunodiffusion.
[c] Statistical significance of differences between groups of isolates from patients with acute versus chronic osteomyelitis, Fisher exact test.
[d] These NT isolates exhibited positive PCR amplification for *cap5*-specific genes.
[e] These NT isolates exhibited positive PCR amplification for *cap8*-specific genes.

aureus isolates from eight different patients suffering from chronic or acute *S. aureus* osteomyelitis, which resulted in the recovery of different *S. aureus* CP phenotypes from the same patients.

MATERIALS AND METHODS

Bacterial isolates. Individual *S. aureus* isolates were obtained from 118 patients with osteomyelitis at seven hospitals in Argentina (four in Buenos Aires City, two in Buenos Aires Province, and one in the City of Santa Fe) to assess the prevalence of encapsulated (CP5 and CP8) and NT *S. aureus*. Sixty-six of these patients suffered from chronic osteomyelitis, 33 suffered from acute osteomyelitis, and the type of osteomyelitis (acute or chronic) remained undefined in 19 patients. The criteria for the diagnosis of chronic osteomyelitis described by Cierny and Mader (9) were used. According to these criteria, the hallmarks of chronic osteomyelitis include a nidus of infected dead bone or scar tissue, an ischemic soft tissue envelope, and a refractory clinical course. In the present study, patients with chronic osteomyelitis were infected for periods exceeding 6 months (range, 6 months to 33 years). Patients with acute infections were infected for <6 months. A total of 50 *S. aureus* isolates were obtained by blood culture from patients at two hospitals in Buenos Aires City with a diagnosis of an acute disease other than osteoarticular infection. In addition, multiple clinical specimens were obtained from seven patients at the Hospital de Clínicas José de San Martín, Universidad de Buenos Aires, Buenos Aires, Argentina, and one patient at the Policlínico Bancario, Buenos Aires, Argentina, in 2006 and 2007. These eight patients suffered from osteomyelitis, and samples of bone and adjacent tissues were obtained for bacteriological evaluation during surgical procedures.

Phenotypic evaluation. Isolation and identification of *S. aureus* were performed according to routine procedures used in the clinical bacteriology laboratory (4). Subcultures of single colonies of homogeneous size and pigmentation from primary isolation agar plates were performed with Trypticase soy agar. Homogeneous colonies from subculture plates were frozen in brain heart infusion-glycerol (20%) until further use. The species was confirmed as *S. aureus* by PCR amplification of species-specific sequences according to Martineau et al. (23) and/or the *S. aureus*-specific *nuc* gene (6). CP5 and CP8 production was assessed by colony immunoblotting and confirmed by immunodiffusion as described elsewhere (20). Isolates yielding no positive precipitation with anti-CP5 or anti-CP8 antibodies in the immunodiffusion test were classified as NT (10). Production of α- and β-hemolysin was assessed by evaluating hemolysis in rabbit and goat blood agar plates, respectively. *S. aureus* RN6390 was used as a positive standard. Slime production was assessed by production of black colonies on Congo red agar (1). Antibiotic susceptibility was tested according to CLSI recommendations. Auxotrophism of a small-colony variant (SCV) isolate was assayed by evaluation of supplemented growth on chemically defined medium agar around disks impregnated with thymidine, hemin, and menadione as described previously (16).

Genotypic analysis. Genomic DNA was extracted from *S. aureus* and purified by a standard procedure (27). The clonality of *S. aureus* isolates was determined by SmaI pulsed-field gel electrophoresis (PFGE) (34) with a CHEF-DR II apparatus (Bio-Rad, Hercules, CA) as previously described (31). The similarity between PFGE types was evaluated by the Dice coefficient. The resultant matrix was analyzed by the unweighted-pair group method using average linkages, and data were analyzed with the TREECON software for Windows. Sequencing of the polymorphic X region of the protein A gene (*spa* typing) was performed by a standard procedure as described previously (19). The presence of the *cap* genes that define the CP5 and CP8 serotypes (*cap5H* to *-J* and *cap8H* to *-K*, respectively) was ascertained by PCR amplification with primers described elsewhere (36). *S. aureus* Reynolds (CP5) and Becker (CP8) were used as positive standards.

Statistical analysis. The distribution of strains in the different experimental groups was analyzed by the Fisher exact test. *P* values of <0.05 were considered significant. Prism 4.0 software (GraphPad) was used for all calculations.

RESULTS

Higher prevalence of NT *S. aureus* in patients with chronic osteomyelitis. Assessment of CP expression on 118 *S. aureus* isolates from patients with osteomyelitis revealed that only 76 (64%) of the isolates expressed CP (57 CP5 and 19 CP8) whereas all 50 isolates from blood cultures of patients with infections other than osteoarticular infections expressed CP (33 CP5 and 17 CP8) (Table 1). The proportion of $CP5^+/CP8^+$ *S. aureus* isolates from patients with osteomyelitis did not differ significantly from that of isolates recovered by blood culture. Evaluation of 99 isolates from patients with precise diagnosis (chronic or acute osteomyelitis) revealed a significantly higher prevalence of NT *S. aureus* in patients with chronic osteomyelitis (53%) than in those with acute osteomyelitis (21%) (Table 2). Amplification analysis of the *cap* genes that determine the CP serotype specificity revealed that 24 (57%) NT *S. aureus* isolates carried the *cap5* genes whereas 18 (43%) of the isolates carried the *cap8* genes. The higher proportion of NT isolates in patients with chronic infections was still significant when the analysis was performed within groups of isolates bearing the *cap5* or the *cap8* genes (Table 2).

Multiple *S. aureus* isolates from selected chronically infected patients. A number of *S. aureus* isolates were obtained from multiple samples of patients with chronic or acute osteomyelitis. Results from each patient are described separately below and in Table 3. The order in which patients are described in the text follows the importance of the findings in each group of *S. aureus* strains under scrutiny. The first six patients (identified as no. 85, 92, 94, 95, 107, and 110) suffered

TABLE 3. Forty-one *S. aureus* isolates from multiple specimens from selected patients with chronic or acute osteomyelitis[a]

Osteomyelitis and strain	Sample	α-Hemolysin	β-Hemolysin	CP serotype	*cap* allele	Slime production[e]	Susceptibility to methicillin[f]	Pulsotype[b]	*spa* type
Chronic									
85a	Bone (purulent secretion)	+	+	CP8	*cap8*	+	S	D	t189
85b	Bone (tibia, distal)	+	+	CP5	*cap5*	+	R	A9	t149
85c	Fibrous soft tissue	−	−	NT	*cap8*	+	S	D	t189
85d	Bone (tibia, proximal)	+	+	NT	*cap5*	+	R	A9	t149
85e	Bone (transplant)	−	−	NT	*cap8*	+	S	D	t189
85f	Bone (tibia)	−	−	NT	*cap8*	+	S	D	t189
Chronic									
92a	Soft tissue adjacent to bone	−	−	CP8	*cap8*	+	S	C	t065
92b	Cranial bone	+	+	CP8	*cap8*	+	S	C	t065
92c	Cranial plate	−	−	CP8	*cap8*	++	S	C	t2271
Chronic									
94a	Hip bone	+	+	CP5	*cap5*	+++	R	A7	t149
94b	Bone cement	+	+	CP5	*cap5*	+++	R	A7	t149
94c	Soft tissue adjacent to bone	+	−	CP5	*cap5*	+++	R	A7	t149
94d	Fistulous purulent secretion	−	−	NT	*cap5*	+++	R	A7	t149
Chronic									
95a	Articular fluid	+	+	NT	*cap8*	+	S	E1	t2271
95b	Bone (astragalus)	+	+	NT	*cap8*	+	S	E1	t2271
95c	Bone (tibia)	+	+	NT	*cap8*	+	S	E1	t2271
Chronic									
107a	Bone cement	−	−	CP5	*cap5*	−	R	A2	t3946
107a1[c]	Bone cement	−	−	CP5	*cap5*	+++	R	A3	t149
107b	Bone (femur)	+	+	CP5	*cap5*	−	R	A8	t149
107c	Soft tissue adjacent to bone	−	−	NT	*cap5*	+++	R	A5	Undefined[d]
107d	Hip purulent secretion	−	−	CP5	*cap5*	+++	R	A4	t149
107e	Cotyloid cement	+	+	CP5	*cap5*	+	R	A4	t149
107f	Cotyloid membrane	−	−	CP5	*cap5*	+++	R	A6	t149
Chronic									
110a	Bone membrane (tibia)	−	−	NT	*cap8*	+++	S	E2	t3796
110b	Bone membrane (femur)	−	−	NT	*cap8*	+++	S	E3	t3796
110c	Bone cement	−	−	NT	*cap8*	+++	S	E3	t3796
110d	Bone (femur)	−	−	NT	*cap8*	+++	S	E3	t3796
Acute									
100a	Hip purulent aspirate	+	+	CP5	*cap5*	+	S	B	t002
100b	Cotyloid membrane	+	+	CP5	*cap5*	+	S	B	ND[g]
100c	Bone cement	+	+	CP5	*cap5*	+	S	B	ND
100d	Soft tissue adjacent to bone	+	+	CP5	*cap5*	+	S	B	ND
Acute									
111a	Superficial secretion	+	+	CP5	*cap5*	+	R	A1	t311
111b	Superficial secretion	+	+	CP5	*cap5*	+	R	A1	ND
111c	Superficial secretion	+	+	CP5	*cap5*	+	R	A1	ND
111d	Superficial secretion	+	+	CP5	*cap5*	+	R	A1	ND
111e	Superficial secretion	+	+	CP5	*cap5*	+	R	A1	ND
111f	Foot secretion	+	+	CP5	*cap5*	+	R	A1	ND
111g	Deep secretion	+	+	CP5	*cap5*	+	R	A1	ND
111h	Deep secretion	+	+	CP5	*cap5*	+	R	A1	ND
111i	Bone (tibia) secretion	+	+	CP5	*cap5*	+	R	A1	ND
111j	Bone (fibula) secretion	+	+	CP5	*cap5*	+	R	A1	ND

[a] All of the strains produced positive PCR amplification for sequences specific for *S. aureus* and positive amplification for *nuc*. These 41 isolates are different from the 99 described in Table 2.
[b] Pulsotype denomination arbitrarily defined for this paper.
[c] Isolate 107a-1 exhibited the SCV phenotype.
[d] DNA extracted from strain 107c did not yield any PCR amplification product with the primers routinely used for *spa*. It did, however, yield PCR amplification of specific *S. aureus* sequences (17).
[e] Levels of slime production: −, absent; +, light; ++, moderate; +++, heavy.
[f] S, susceptible; R, resistant.
[g] ND, not done.

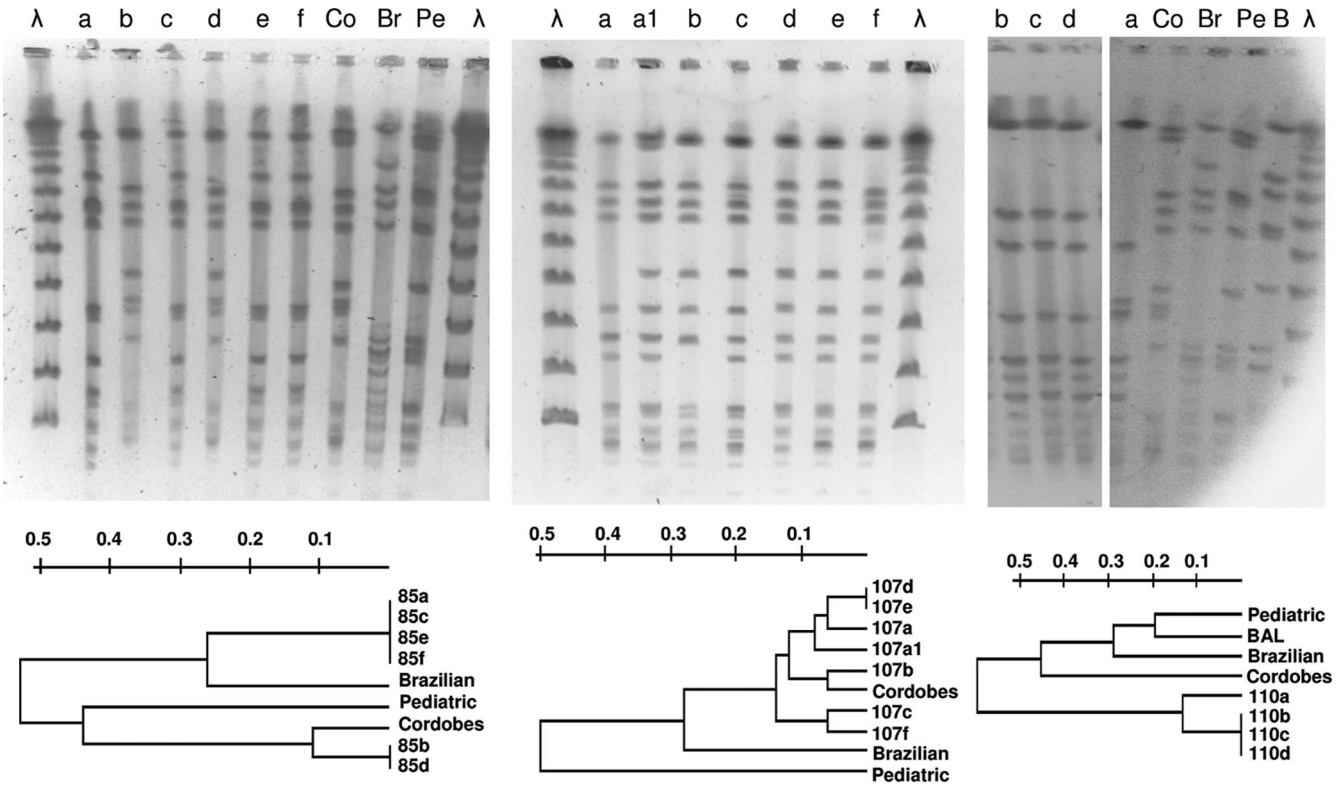

FIG. 1. SmaI PFGE band patterns of *S. aureus* strains isolated from patients 85 (left panel), 107 (center panel), and 110 (right panel). Lanes λ, lambda ladder. Dendrograms show the similarity among isolates from each patient. Representatives of the prevalent Brazilian (Br), Pediatric (Pe), and Cordobes (Co) MRSA clones in Argentina were included in the analysis. BAL is a representative strain of a new emerging MRSA clone in Buenos Aires, obtained from a respiratory specimen of a child with cystic fibrosis (Liliana Jordá Vargas, personal communication).

from chronic osteomyelitis, whereas the remaining two (no. 100 and 111) suffered from acute osteomyelitis.

(i) Isolates from patient 85. Specimens were obtained from a 20-year-old man with chronic osteomyelitis of the right tibia (Table 3). *S. aureus* 85a was isolated from a fistulous purulent secretion. Thirteen months later, four samples obtained during a right tibial surgical debridement yielded a culture positive for *S. aureus* (isolates 85b through 85e). Finally, 4 months afterward, one last *S. aureus* isolate (85f) was recovered from a specimen from the infected tibia. Strains 85a, 85c, 85e, and 85f belonged to *spa* type t189; exhibited the same pulsotype, D (Fig. 1, left panel); were susceptible to methicillin; and produced small amounts of slime (Table 3). Strain 85a was $CP8^+$ and produced α- and β-hemolysin, whereas strains 85c, 85e, and 85f were NT and did not express α- and β-hemolysin. These three NT strains carried the *cap8* genes, and $CP8^+$ strain 85a had been isolated 13 months before strains 85c, 85e, and 85f. These results indicate that these three NT strains may have derived from $CP8^+$ strain 85a during infection due to a mutation in a regulatory gene (10). On the same day that strains 85c and 85e were recovered, strains 85b and 85d were isolated from two other separate specimens (Table 3). Strains 85b and 85d exhibited the same *spa* type (t149) and pulsotype (A9), were resistant to methicillin (Fig. 1, left panel), and were mild slime producers. Strain 85b was $CP5^+$, whereas strain 85d was NT. These results plus the facts that both 85b and 85d produced α- and β-hemolysin and that NT strain 85d carried the *cap5* genes indicate that NT strain 85d may have derived from strain 85b due to a mutation not involving a regulatory gene. Other conclusions from patient 85 strains are that (i) the selection of NT *S. aureus* was irrespective of the CP serotype involved, (ii) strains of distinct phenotypes and genotypes can be isolated from the same patient, and (iii) concomitant infection of the patient with different clones of *S. aureus* (methicillin-resistant *S. aureus* [MRSA] and methicillin-susceptible *S. aureus*) can occur during chronic infection.

(ii) Isolates from patient 107. Six *S. aureus* isolates identified as strains 107a through 107f were obtained from six samples taken from a 74-year old woman with prosthesis-associated chronic fistulous osteomyelitis of the femur (Table 3). All of these strains displayed the same typical colony morphology and pigmentation following incubation for 24 h at 37°C. All of the *S. aureus* strains from patient 107 expressed CP5, except one strain (107c), which was NT. Strain 107c carried the *cap5* genes and exhibited a pulsotype (A5) of high homology (96%) to those of isolates 107a, 107d, 107e, and 107f (Fig. 1, center panel). There was no correlation between CP5 and hemolysin production. Indeed, $CP5^+$ non-SCV phenotype strains 107b and 107e produced both α- and β-hemolysin whereas $CP5^+$ strains 107a, 107d, and 107f did not express hemolysin activity. These results suggest that the genetic lesion responsible for the 107c strain's lack of CP5 expression may have not been a mutation in a main regulatory gene and that 107c may have evolved from 107a, 107d, or 107f or that strains 107a, 107c,

107d, and 107f may have evolved from a close $CP5^+$ common ancestor. After primary culture of the bone cement sample for 72 h, small, nonhemolytic, nonpigmented colonies were detected in addition to a normal phenotype strain (namely, 107a). These tiny colonies (named strain 107a-1) failed to grow on mannitol-salt agar and yielded a negative coagulase reaction at 4 h but a positive reaction at 18 h. Positive amplification with *S. aureus*-specific sequences (23) suggested that these colonies represent SCVs (29). The isolate was resistant to gentamicin (MIC = 6 μg/ml), as the remaining six *S. aureus* isolates obtained from this patient (MICs of 6 to 10 μg/ml). The SCVs retained this phenotype by the seventh passage on blood agar and later started to revert to the normal phenotype. Further characterization of the strain 107a-1 SCV phenotype revealed auxotrophism to hemin (39). Although strains 107a and 107a-1 (SCV) were isolated from the same primary culture plate, they exhibited different PFGE band patterns (ca. 96% similarity). The data also suggest that strain 107c may have not resulted from superinfection with an exogenous strain. The PFGE band patterns and the genetic relatedness of these isolates of the seven strains from patient 107 are depicted in Fig. 1 (center panel). Only two of the seven strains were not discriminated by PFGE analysis (107d and 107e) but were differentiated by the production of α- and β-hemolysin (Table 3). All seven strains exhibited a similarity of greater than 88% and therefore were considered subtypes of the same pulsotype (Table 3). All of the strains from patient 107 were resistant to methicillin and were *spa* type t149, except strain 107c, which yielded negative PCR amplification with *spa* and therefore could not be *spa* typed by the routine procedure. The seven *S. aureus* strains showed different levels of slime production.

(iii) Isolates from patient 110. Four *S. aureus* isolates were obtained from four specimens taken during the surgical debridement of a 79-year-old woman with prosthesis-associated chronic osteomyelitis of the left knee (Table 3). The isolates (110a through 110d) were NT, bore the *cap8*-specific allele, did not express hemolysins, were susceptible to methicillin, and were heavy slime producers. Strains 110b, 110c, and 110d presented the same pulsotype (E3) (Table 2 and Fig. 1, right panel). Strain 110a (pulsotype E2) exhibited only 78% homology to strains 110b, 110c, and 110d. All four strains from patient 110 belonged to the t3796 *spa* type group.

(iv) Isolates from patient 94. Four *S. aureus* isolates were obtained from a 69-year-old woman with prosthesis-associated chronic osteomyelitis of the left hip (Table 3). Strains 94a, 94b, and 94c expressed CP5, whereas strain 94d was NT. The NT strain bore the *cap5*-specific allele. The four strains from patient 94 were genealogically indistinguishable from each other, as ascertained by their pulsotype (PFGE gel not shown) and *spa* type (t149); were resistant to methicillin; and were heavy slime producers. Two isolates expressed hemolysins (94a and 94b), whereas another isolate (94d) did not. Strain 94c expressed α-hemolysin but did not express β-hemolysin (Table 3). These results suggest that NT strain 94d may have emerged as a result of mutation in a regulatory gene (9).

(v) Isolates from patient 92. Three *S. aureus* isolates were obtained during the surgical debridement of a 45-year-old man with prosthesis-associated chronic cranial osteomyelitis (Table 3). These strains expressed CP8, but only strain 92b expressed hemolysins. The three strains exhibited the same pulsotype (C), but strain 92c belonged in a *spa* type group (t2271) different from that of strains 92a and 92b (t065). The three isolates were susceptible to methicillin and exhibited little or moderate slime production.

(vi) Isolates from patient 95. Three *S. aureus* isolates were obtained from three specimens taken during the surgical debridement of a 71-year-old man with prosthesis-associated chronic osteomyelitis of the ankle bones (Table 3). There were no phenotypic differences among isolates 95a through 95c. The three strains from patient 95 were NT, carried the *cap8* allele, exhibited the same pulsotype (E1) and *spa* type (t2271), produced a small amount of slime, and were susceptible to methicillin.

Multiple *S. aureus* isolates from selected acutely infected patients. (i) Isolates from patient 100. Four *S. aureus* isolates were obtained from four specimens taken during surgery from a 72-year-old woman (patient 100) with prosthesis-associated acute osteomyelitis of the hip bone (Table 3). These *S. aureus* strains (100a through 100d) showed no phenotypic differences from each other, and all expressed CP5. These four strains exhibited the same pulsotype (B). Only strain 100a was *spa* typed (t0002). No differences in the level of slime production were found, and all four isolates were susceptible to methicillin.

(ii) Isolates from patient 111. Ten *S. aureus* isolates were obtained from 10 specimens taken during surgery from a 14-year-old boy with acute osteomyelitis of the ankle bones involving the fibula and tibia (Table 3). There were no phenotypic differences among the 10 isolates (111a through 111j). The 10 *S. aureus* strains from patient 111 expressed CP5 and exhibited the same pulsotype (A1). The *spa* type of strain 111a was determined (t0002). No differences in the level of slime production were found, and all 10 isolates were resistant to methicillin.

DISCUSSION

S. aureus CP5 or CP8 is produced by ~80% of human isolates (15). Evidence that CP5 and CP8 promote staphylococcal virulence has come from in vitro experiments, as well as from studies performed with diverse animal models of *S. aureus* infection and colonization. The experimental studies performed and the major results obtained are described in recent reviews by O'Riordan and Lee (26) and Lee and Lee (22). Loss of capsule expression, however, may play a role in *S. aureus* persistence in the chronically infected host (8, 35, 36). It has also been shown that NT *S. aureus* is noticeably prevalent in bovines with subclinical mastitis (long-term infection) and could be as high as 86% (33). Here we demonstrate that NT *S. aureus* is more frequently isolated from patients with chronic osteomyelitis than from those with acute osteomyelitis. The role of CP in acute invasive infection was corroborated by the finding that all of the *S. aureus* isolates obtained by blood culture from patients with diseases other than osteomyelitis expressed either CP5 or CP8.

Studies performed with an animal model of mammary infection and with cultures of a bovine epithelial cell line led to the conclusion that loss of capsule expression may give *S. aureus* an advantage to persist intracellularly (8, 35, 37). The analysis of *S. aureus* isolates from different specimens obtained

from the six patients with chronic osteomyelitis described in this report revealed that, except for one (patient 92), all of them had at least one specimen culture positive for NT *S. aureus*. The pulsotypes of the NT isolates were identical or exhibited high homology to those of encapsulated (CP5 or CP8) *S. aureus* isolated from the same patient, suggesting a common ancestor. In one patient (no. 85), an encapsulated *S. aureus* strain was isolated from an infected bone and in another specimen from the same patient obtained 13 months later, an *S. aureus* isolate of the same pulsotype and *spa* type was obtained, but this time it exhibited an NT phenotype. Conversely, two patients with acute osteomyelitis harbored only encapsulated *S. aureus* of homogeneous pulsotypes (4 and 10 isolates each, pulsotypes B and A1, respectively). Furthermore, *S. aureus* isolates obtained from the blood of 50 patients with acute disease were all encapsulated. Taken together, our results permit two concurrent hypotheses, one from the evolutionary standpoint and another from the pathogenesis standpoint, to explain the mechanism leading to the high prevalence of NT *S. aureus* in patients with chronic osteomyelitis.

From the evolutionary standpoint, it can be hypothesized that long-term infection is required for selection of NT *S. aureus*. This hypothesis involves the concept that NT strains are the consequence of long-term chronic osteomyelitis. From our results, it became apparent that multiple *S. aureus* isolates from patients suffering from chronic osteomyelitis over long periods of time displayed, in most cases, phenotypic and genotypic diversity. In contrast, isolates from two acutely infected bone and adjacent tissue samples were totally homogeneous in phenotype and genotype. These results support the hypothesis that *S. aureus* adapts to continuously changing microenvironments in bone and adjacent soft tissue, resulting in the selection of strains with distinct phenotypic features in the chronically infected host. Similar diversity in the *S. aureus* population was found by Goerke et al. in sputum samples from cystic fibrosis patients who are chronically infected with *S. aureus* (12). Goerke et al. (12) explained their results, as we can explain ours, by the "insurance hypothesis," which predicts that a more diverse bacterial community will be better able to resist external stress (5). Therefore, the ability of *S. aureus* to adapt to changing conditions in the invaded tissue microenvironments during chronic infection would be the key mechanism that permits persistence in the infected host. Many factors present in infected tissues may promote the selection of a bacterial phenotype better adapted for persistence and may be responsible for the diversity of the *S. aureus* strains isolated from different niches of the same chronically infected patient at the same time. These factors include varying concentrations of CO_2 and iron, redox potential, nutrient availability (26), and antimicrobial agents (17), among others. Only two factors, however, have been clearly demonstrated to pose selective pressure for an *S. aureus* persistent phenotype. These are antibodies to CP (37), which select for NT variants and SCVs, and gentamicin, which selects for SCVs (3, 7). *S. aureus* SCVs represents a subpopulation that exhibits, among other phenotypic features, a characteristic slow-growth phenotype (30). The clinical importance of *S. aureus* SCVs as causative agents facilitating persistent and recurrent infections which are refractory to antibiotic treatment has been recognized (29, 30). Osteoarticular tissues (38), as well as the lungs of cystic fibrosis patients (17), have been shown to provide excellent niches with microenvironmental conditions adequate for the selection of *S. aureus* variants (NT strains and SCVs) which infect and persist over extremely long periods.

From the pathogenesis standpoint, it can also be hypothesized that the lack of capsule or loss of CP expression promotes the establishment of chronic, long-term osteomyelitis. This hypothesis involves the speculation that the emergence of NT strains is the cause of chronic osteomyelitis, not the consequence. It is known that successful adaptation of *S. aureus* to the host is achieved by regulatory mechanisms in the short term and by inheritable shifts in the population over the long term (12). At the early stages of infection, CP may be required by *S. aureus* to invade healthy tissue. Replication of *S. aureus* would provoke changes in the infected tissue, thus creating microenvironments responsible for distinct signals, which would be recognized by the bacteria. The *S. aureus cap5/8* locus is under the control of an extremely complex regulatory network (22, 26). Whether these changing signals downregulate the production of CP in vivo in infected bone remains unknown and requires further research. Several studies have demonstrated the expression of CP in vivo (13, 18, 21, 28). Minimal expression of CP5 was observed, however, in tissues obtained from cystic fibrosis patients chronically infected with *S. aureus* (14, 24). Downregulation of CP expression may generate bacteria with unstable NT phenotypes that are better able to reach the intracellular milieu through a surface adhesin-receptor interaction than are encapsulated bacteria. These regulatory NT variants plus other newly generated stable NT mutants may be the key to persistence. In the presence of antibodies to CP and over a long period of time, stable NT *S. aureus* would start to emerge at the infection site (37). Beyond the mechanisms responsible for loss of CP expression (inheritable or regulatory), NT *S. aureus* bacteria can more easily reach the intracellular milieu (8, 35, 37), where they would avoid further removal by antibodies and persist. Our results support both hypotheses (from the evolutionary and pathogenesis standpoints) since it is equally true that the NT phenotype helps *S. aureus* to become intracellular and persist and that simultaneously long-term infection promotes the selection of NT *S. aureus*.

Finally, our results also raise the issue of the clinical impact of phenotypic differences in multiple *S. aureus* isolates from a single infected patient. Whereas criteria for the bacteriological diagnosis of bone infection are generally accepted (2, 11), the phenotypic differences found in multiple *S. aureus* isolates from a patient with chronic osteomyelitis may have relevance for antimicrobial susceptibility testing. Indeed, in this study, MRSA and methicillin-susceptible *S. aureus* were isolated from different specimens of the same chronically infected patient. Therefore, in order to ensure selection of the adequate antimicrobial agent for a patient with a chronic bone infection, susceptibility testing should be performed on isolates representative of each specimen with an *S. aureus*-positive culture.

In conclusion, we demonstrated that NT *S. aureus* is more frequently isolated from patients with chronic osteomyelitis

than from those with acute osteomyelitis, suggesting that loss of CP expression may be advantageous to *S. aureus* during chronic infection. Our study of multiple *S. aureus* isolates from individual patients allowed us to suggest that loss of CP expression is likely to have occurred in the patient during long-term bone infection, although one case of reinfection with a different clone of *S. aureus* was found. An additional observation was that representatives of a homogeneous population of *S. aureus* can be isolated from multiple clinical samples from patients with acute osteomyelitis. Conversely, a mostly heterogeneous *S. aureus* population composed of strains with dissimilar phenotypic features and potential different diagnostic relevance was found in multiple specimens from the same patient with chronic osteomyelitis, suggesting the need for adequate guidelines for the clinical bacteriological diagnosis of these patients.

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