

Hypervariability of Biofilm Formation and Oxacillin Resistance in a *Staphylococcus epidermidis* Strain Causing Persistent Severe Infection in an Immunocompromised Patient[∇]

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We report on a leukemic patient who suffered from a persistent, generalized, and eventually fatal *Staphylococcus epidermidis* infection during prolonged aplasia. Over a 6-week period, we isolated a genetically and phenotypically unstable *S. epidermidis* strain related to an epidemic clone associated with hospital infections worldwide. Strikingly, the strain showed a remarkable degree of variability, with evidence of selection and increasing predominance of biofilm-producing and oxacillin-resistant variants over time. Thus, in the early stages of the infection, the strain was found to generate subpopulations which had spontaneously lost the biofilm-mediating *ica* locus along with the oxacillin resistance-conferring *mecA* gene. These deletion mutants were obviously outcompeted by the *ica*- and *mecA*-positive wild-type genotype, with the selection and predominance of strongly biofilm-forming and oxacillin-resistant variants in the later stages of the infection. Also, a switch from protein- to polysaccharide intercellular adhesin/poly-*N*-acetylglucosamine (PIA/PNAG)-mediated-biofilm production was detected among *ica*-positive variants in the course of the infection. The data highlight the impact of distinct *S. epidermidis* clonal lineages as serious nosocomial pathogens that, through the generation and selection of highly pathogenic variants, may critically determine disease progression and outcome.

Coagulase-negative staphylococci (CoNS) contribute significantly to catheter-related bloodstream infections after hematopoietic stem cell transplantation (HSCT) (15). Recent studies identified defined clonal lineages for CoNS that persist in health care units and might spread even within or between hospitals (3, 8, 19). Various multilocus sequence typing (MLST) studies have identified an epidemic *Staphylococcus epidermidis* clone, named sequence type ST27 (or, alternatively, ST2, according to a revised MLST scheme) (12), as the cause of a majority of nosocomial *S. epidermidis* infections in numerous hospital settings (8, 10, 12, 20). In *S. epidermidis*, biofilm formation has been recognized as a crucial factor for the colonization of medical devices (reviewed in references 13 and 14). Biofilms can be generated by various mechanisms, and polysaccharide intercellular adhesin/poly-*N*-acetylglucosamine (PIA/PNAG)-mediated-biofilm formation is a very common one (13). The enzymes that facilitate PIA/PNAG synthesis are

encoded by the four-gene operon *icaADBC*, which is present in many disease-associated *S. epidermidis* strains, including ST27/ST2 strains (9, 10). Here, we report on a leukemic patient who received an allogeneic HSCT and experienced a generalized *S. epidermidis* infection. We describe the affiliation of the strain to the epidemic ST27/ST2 clone and investigate genomic instability and variations in biofilm expression and oxacillin resistance that occurred in the course of the infection.

Case Report

A 23-year-old male patient was treated with an allogeneic cord-blood HSCT for a second relapse of a high-risk common-B-cell acute lymphoblastic leukemia. On day 5 posttransplant, the patient became febrile. Piperacillin-tazobactam treatment was started empirically and, when the fever persisted, was supplemented with caspofungin treatment on day 13. *S. epidermidis* grew in three of the four blood culture bottles (Table 1) (isolates 1 and 2). The central venous line was removed.

The patient was treated with vancomycin (2 × 1 g intravenously [i.v.]) for 10 days. Blood cultures were negative during treatment. Five days (day 29) after vancomycin treatment was stopped, he developed bacteremia again; four of four bottles were positive for *S. epidermidis* (Table 1) (isolates 3 and 4). The central venous line was removed, and i.v. treatment with vancomycin and catheter-lock therapy was begun. Blood cultures remained positive for *S. epidermidis* (Table 1) (isolates 5 to 8); because of low trough levels of vancomycin (i.e., 4.4

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TABLE 1. Microbiological and genetic properties of *S. epidermidis* isolates

Isolate sample	Day after transplantation	Sample source	Biofilm formation ^a	Result for <i>icaA</i> and <i>icaC</i> PCR	Oxacillin susceptibility ^b	Result for <i>mecA</i> PCR	SCC _{mec} type	MLST	No. of IS256-specific fragments
1	13	Blood culture	Negative	Negative	S	Negative	VI ^c	ST27/ST2 ^d	4
2		Blood culture	Positive (protein)	Positive	R	Positive	VI	ST27/ST2	5
3	29	Blood culture	Negative	Negative	S	Negative	VI ^c	ST27/ST2 ^d	4
4		Blood culture	Positive (protein)	Positive	R	Positive	VI	ST27/ST2	5
5	34	Blood culture	Positive (protein)	Positive	R	Positive	VI	ST27/ST2	5
6		Blood culture	Negative	Negative	S	Negative	VI ^c	ST27/ST2 ^d	4
7	40	Blood culture	Positive (protein)	Positive	R	Positive	VI	ST27/ST2	5
8		Blood culture	Negative	Negative	S	Negative	VI ^c	ST27/ST2 ^d	4
9	52	Blood culture	Strongly positive (PIA)	Positive	R	Positive	VI	ST27/ST2	5
10		Blood culture	Strongly positive (PIA)	Positive	R	Positive	VI	ST27/ST2	5
11	55	CSF	Strongly positive (PIA)	Positive	R	Positive	VI	ST27/ST2	5
12		CSF	Strongly positive (PIA)	Positive	R	Positive	VI	ST27/ST2	5

^a "PIA" and "protein" indicate PIA-mediated biofilms and protein-mediated biofilms, respectively.

^b S, sensitive; R, resistant.

^c Isolates that displayed an SCC_{mec} type-VI pattern, but for which the *mecA* gene was not detected.

^d Isolates that lacked the *arcC* gene, which is part of both MLST schemes.

µg/ml), the dosage was increased to 2×1.5 g. The central line was replaced again. The patient then developed a painful bilateral swelling above the tibial muscles with redness and tenderness. The level of creatinine kinase increased from 21 U/liter to 400 U/liter. Magnetic resonance imaging (MRI) revealed TH2 hyperintensity along the *musculus peroneus longus* and *musculus extensor hallucis*, with collection of fluid in the muscle, but no abnormalities of the fascias, favoring the diagnosis of myositis. A repeat MRI 5 days later showed involvement of the anterior and posterior tibial muscles (data not shown).

On day 42, still in aplasia, histological results from biopsies at different sites in both tibial muscles showed necrosis and clusters of coccoid bacteria. The culture yielded *S. epidermidis*. Amikacin was added to vancomycin. Daily blood cultures then were sterile until day 52, when another blood culture yielded *S. epidermidis* (Table 1) (isolates 9 and 10). On day 55, the patient complained of a headache and presented nuchal rigidity. An MRI of the brain showed two 4- to 6-mm size-enhancing lesions in the prefrontal cortex, which is consistent with the presence of septic emboli. A spinal tap produced a clear cerebrospinal fluid (CSF) that had a slightly elevated cell count (5.3×10^6 cells/liter), a protein load of 576 mg/liter, and the presence of *S. epidermidis* (Table 1) (isolates 11 and 12). Because of its good penetration into the central nervous system, rifampin (2×600 mg i.v.) was added to vancomycin, and treatment with amikacin was stopped. After 70 days of non-engraftment, a haplo-identical rescue transplantation from the mother was performed. The patient remained febrile, but daily blood cultures were negative. On day 72, the patient developed acute respiratory failure with rapidly progressive acute respiratory distress syndrome (ARDS). Despite mechanical ventilation and extracorporeal oxygenation, the patient died 2 days later of multiorgan failure. The autopsy did not reveal any sign of leukemia or any sign of endocarditis.

MATERIALS AND METHODS

Strain cultivation and characterization. Samples were cultured on blood agar plates, and representative colonies from various morphotypes were selected and analyzed by standard microbiological techniques and 16S rRNA gene sequencing, respectively. PCR detection of the *icaA* and *icaC* genes was performed as described previously (21).

Biofilm testings. Biofilm formation was tested on 96-well, polystyrene tissue culture plates as described previously (21), using tryptone soya broth (TSB; Oxoid) as a growth medium (17.0 g/liter pancreatic digest of casein, 3.0 g/liter papaic digest of soybean meal, 5.0 g/liter sodium chloride, 2.5 g/liter dipotassium hydrogen phosphate, 2.5 g/liter glucose). *S. epidermidis* RP62A and *Staphylococcus carnosus* TM300 were used as positive and negative controls, respectively. To determine whether the biofilm was mediated by PIA production or by proteins, biofilms were treated with sodium periodate and proteinase K, respectively (7).

In vitro detection of biofilm-negative variants. Generation of biofilm-negative variants *in vitro* was established using Congo red agar (CRA) as a biofilm indicator medium (4), and frequencies were calculated as described previously (21). Briefly, a single bacterial colony was picked and diluted in phosphate-buffered saline. Appropriate aliquots of this dilution were used to inoculate 50 ml tryptone soya broth, and CFU in the inoculum were determined by plating and incubating the corresponding aliquots on tryptone soy agar. Following incubation overnight at 37°C with agitation, the optical density of the liquid culture was determined and serial dilutions were plated onto CRA. Upon incubation for 18 h at 37°C and at least another 12 h at room temperature, biofilm-forming colonies appear on CRA as black colonies, while biofilm-negative variants are red. Total CFU on the plates as well as black and red colonies were counted, and the frequency of the occurrence of biofilm-negative variants per cell and generation was calculated using the equation $P = 1 - n\sqrt{1-x}$, in which x is defined as the number of red colonies divided by the number of total CFU. n represents the number of generations which are determined by the equation $n = (\log N - \log N_0)/(\log 2)$, where N_0 is the number of bacterial cells in the inoculum and N is the total number of bacterial cells in the liquid culture after incubation.

DNA fingerprinting by PFGE. Genomic DNA for pulsed-field gel electrophoresis (PFGE) was prepared and resolved in a CHEF-DR III apparatus as described previously (5).

MLST. MLST was performed as described previously (20) and by using the revised scheme according to Miragaia et al. (12).

SCC_{mec} typing and IS256 detection. Staphylococcal cassette chromosome *mec* (SCC_{mec}) typing and *mecA* PCR were performed as previously described (11). To detect IS256 copies, chromosomal DNA was isolated and then EcoRI digested, blotted, and hybridized with an IS256-specific gene probe (21).

RESULTS

Primary cultivation of blood and CSF samples resulted regularly in the appearance of colonies differing in size and color. They were picked and identified as *S. epidermidis* by 16S rRNA sequencing. Antibiotic resistance analyses and PCR detection of the methicillin/oxacillin resistance-mediating *mecA* gene revealed the simultaneous presence of both oxacillin-resistant/*mecA*-positive and oxacillin-susceptible/*mecA*-negative colonies in blood culture samples for days 13 to 40, while at a later stage of infection (days 52 and 55), isolates were uniformly oxacillin resistant (Table 1).

The *mecA* gene is usually located on SCC*mec* cassettes that represent large, structurally diverse, integrated genomic islands of the staphylococcal chromosome, which can be detected by a multiplex PCR approach (11). SCC*mec* typing identified a type VI cassette in all isolates, but with no *mecA* gene in the oxacillin-sensitive variants (Table 1). Notably, SCC*mec* type VI-specific PCR fragments were still detectable in the *mecA*-negative/oxacillin-sensitive variants, suggesting that the SCC*mec* cassette was lost in part and that the deleted fragment included the *mecA* gene.

Interestingly, differences in oxacillin resistance were clearly associated with various biofilm-forming capacities. Thus, in biofilm-forming tests *in vitro*, *mecA*-negative isolates were found to be biofilm negative, while *mecA*-positive isolates were biofilm positive, which was in good agreement with the detection of the *ica* genes in biofilm-positive isolates (Fig. 1A and Table 1). *ica*- and *mecA*-positive colonies coexisted with *ica*- and *mecA*-negative colonies in samples obtained at the beginning of the infection, whereas later (days 52 and 55), only biofilm-forming, *ica*- and *mecA*-positive colonies prevailed (Fig. 1A). Even more striking was the difference in the amounts of biofilm produced by *ica*-positive isolates. Thus, isolates 2, 4, 5, and 7 (days 13 to 40) generated less biofilm than isolates 9 to 12, which were grown from blood and cerebrospinal fluid samples from a later stage of infection (days 52 and 55). Proteinase K and metaperiodate treatment of the biofilms demonstrated that weaker biofilms were mediated by protein but that the strong biofilms recorded at the end were due to PIA production (Fig. 1A).

As the mobile genetic element IS256 has been reported to be involved in variations of biofilm formation in *S. epidermidis* (1, 21), the isolates were also tested for this element. Southern hybridization of chromosomal DNA revealed the presence of multiple IS256 copies in all isolates, with uniform hybridization patterns within *mecA*- and *ica*-positive and *mecA*- and *ica*-negative variants, but with aberrations between those groups. Thus, the *ica*-positive variants exhibited five IS256-specific bands, whereas *ica*-negative isolates displayed only four such bands (Fig. 1C).

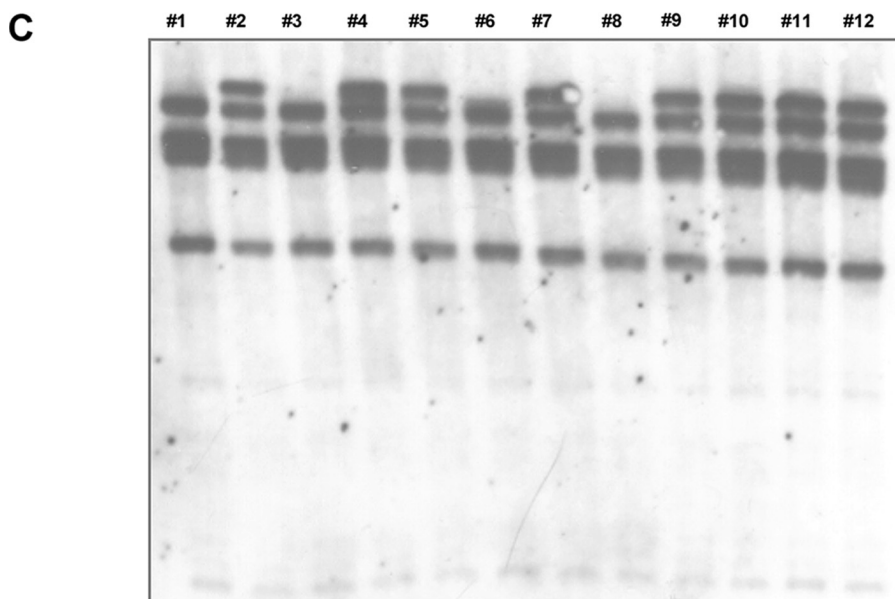
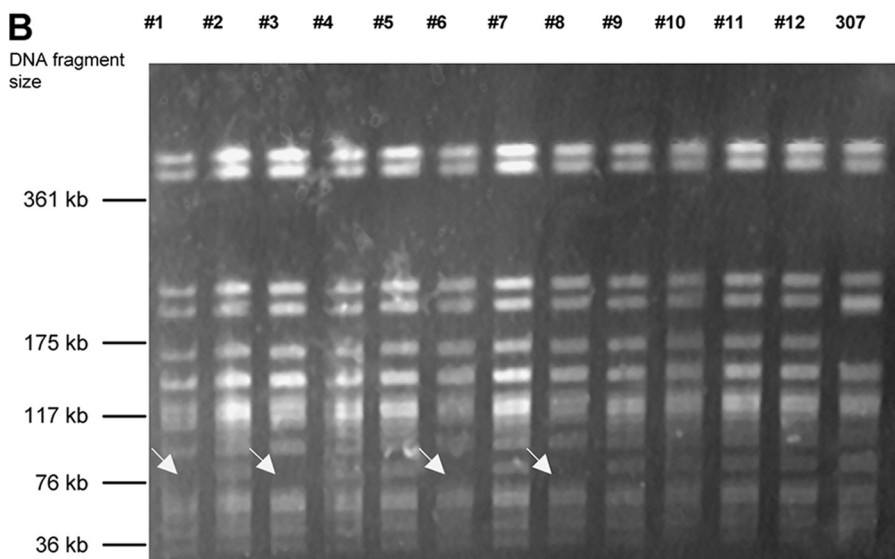
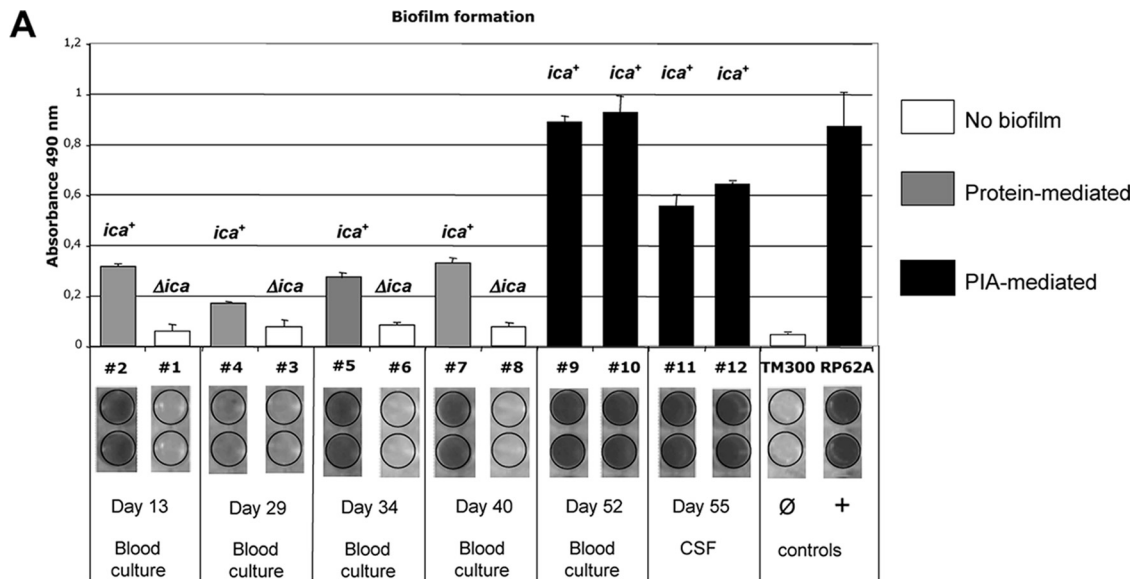
The findings so far strongly suggested a mixed infection by at least two different *S. epidermidis* strains. Molecular typing by PFGE, however, revealed nearly identical restriction patterns pointing to a clonal origin of the isolates (Fig. 1B). The only difference recorded was the regular absence of a 100-kb DNA fragment in *mecA*- and *ica*-negative isolates (identified by arrows in Fig. 1B). When two different MLST schemes were applied, all isolates were identified as ST27 (20) or, alternatively, as ST2 (12), confirming again the clonality of the iso-

lates. It is noteworthy that all *mecA*- and *ica*-negative variants also lacked the *arcC* gene, which is part of both MLST schemes (Table 1). To further establish the clonality of the isolates, we analyzed whether or not *mecA*- and *ica*-negative variants may arise from a *mecA*- and *ica*-positive genotype *in vitro*. For this purpose, we grew the biofilm-forming, *ica*- and *mecA*-positive isolate 2 on Congo red agar, an indicator medium on which biofilm-formers appear as black colonies, while biofilm-negative variants are red. A single black colony was picked, and appropriate dilutions were spread on CRA plates. Following incubation, both black and red colonies arose on the indicator medium, with red colonies occurring at a frequency of 10^{-2} per cell and generation. Biofilm tests as well as *ica*- and *mecA*-specific PCRs demonstrated a biofilm-negative phenotype in the red colonies, which was again accompanied by lack of the *ica* and *mecA* genes, respectively. Finally, the PFGE restriction patterns of the variants were found to be identical to those of the *ica*- and *mecA*-negative deletion mutants detected in the patient (data not shown). In summary, these experiments demonstrate the genomic instability of the *ica* and *mecA* genes as a molecular background for the observed heterogeneity rather than a mixed infection by different strains.

DISCUSSION

S. epidermidis is primarily a commensal bacterium with a low level of pathogenic potential, and often, it is difficult to decide whether a specific isolate is the cause of an infection or represents an unspecific contamination. The appearance of various colony morphotypes in the primary cultures obtained from our patient mimicked a mixed culture, suggesting contamination. However, molecular typing revealed a common clonal origin for all the isolates. The strain was identified as ST27/ST2, an *S. epidermidis* sequence type that is currently emerging as an epidemic nosocomial pathogen in medical facilities worldwide (8, 10, 12, 20). It is tempting to speculate that the "success" and wide distribution of ST27/ST2 strains are linked to the specific genetic makeup of these strains, which may be illustrated by the fact that the vast majority of the strains carry the *ica* operon and are capable of producing biofilms (10). Moreover, these strains seem to acquire and readily exchange mobile genetic elements, such as various SCC*mec* cassettes and IS elements, the latter being important genetic factors associated with genome flexibility (10, 12).

The hypervariable ST27/ST2 *S. epidermidis* strain detected in our patient generated a multitude of phenotypic and genetic variants. The prolonged persistence in the patient's blood cultures and the ensuing medical complications might, at least in part, be explained by bacterial biofilm production, which is often associated with reduced *in vivo* antibiotic susceptibility. Biofilm formation and genetic adaptability are crucial factors employed by *S. epidermidis* to cope with changing environmental conditions, such as antibacterial host response and antibiotic treatment. In our case, biofilm formation and oxacillin susceptibility proved to be variable features. They were associated with the conservation or deletion of the *ica* and *mecA* genes. Interestingly, biofilm and resistance variants coexisted only at the beginning of the infection. Later in infection, *ica*- and *mecA*-negative variants appeared to be outcompeted by strongly biofilm-producing and oxacillin-resistant variants (Fig.



1A). Interestingly, *ica*-positive variants isolated early in infection (between days 13 and 40) produced protein-mediated rather than PIA-mediated biofilms, although the *icaADBC* operon was present in those isolates (Fig. 1A). Preliminary transcriptome studies revealed no significant differences in *icaADBC* operon transcription between the *ica*-positive variants obtained early and late in infection (S. M. K. Schoenfelder and W. Ziebuhr, unpublished data). The molecular basis for the lack of PIA production remains to be elucidated but may be linked to differing metabolic regulation. Also, the factors involved in protein-mediated-biofilm formation at the beginning of the infection are not established yet and require further analysis. Possible candidates for future studies are the accumulation associated protein Aap, the biofilm-associated protein Bhp, and other cell wall-associated proteins known to be involved in biofilm production (13).

Although the molecular details are unknown, recent *in vitro* studies clearly demonstrate that *S. epidermidis* is capable of switching between different modes of biofilm formation (i.e., PIA versus protein) (7). Our data suggest that this mechanism may also have biological relevance in the dynamic process of an infection. It is generally thought that PIA-mediated-biofilm production provides a selective advantage in established, device-associated infection, while representing a loss of fitness when the isolates colonize the skin (2, 16). In line with this idea, competition experiments in a device-related animal model using *ica* mutants and PIA-producing strains revealed growth defects in the *ica* mutants (2). Over time, the mutants were outcompeted by their *ica* wild-type counterparts, giving rise to the selection of strong biofilm formers in the course of the infection (2), which is in perfect agreement with the observed predominance of PIA-producing variants in our patient later in the infection. In contrast, in a human skin colonization model, a PIA-producing variant was shown to be outcompeted by its isogenic *ica* mutant, suggesting that PIA is not required or even detrimental when *S. epidermidis* colonizes the skin (16). In light of these findings, it is tempting to speculate that PIA production might be controlled by the environment that the bacteria encounter and that in our patient, the PIA-negative variants early in infection might have switched off PIA production, as their gene expression status still resembles that of skin colonizers. In an established device-related infection in the bloodstream, PIA might be required and beneficial for escaping the innate immune system and for withstanding the action of antibiotics (13, 18), which would explain the strong PIA production later in infection. However, more experimental work is needed to substantiate this hypothesis and to elucidate the regulatory mechanisms involved in this process.

The genetic mechanisms causing the spontaneous generation of *ica*- and *mecA*-negative variants is an interesting part of the study. The variants were found to lack a large DNA fragment that also includes the *ica* genes and part of the *SCCmec*

type VI cassette (including *mecA*) (Fig. 1B and Table 1). Interestingly, these deletions could also be triggered *in vitro*, where they occurred at a surprisingly high frequency of 10^{-2} per cell and generation. Typically, *S. epidermidis* biofilm variations are known to occur in the order of magnitude of 10^{-5} to 10^{-6} per cell and generation and were found to be associated with classical phase variation events, mostly by inactivation of biofilm genes or their corresponding regulators (1, 6, 21). Deletion of large genomic DNA fragments at such a high frequency is a novel phenomenon, and at the present stage of experimental work, we have no compelling evidence for an underlying mechanism. However, the deletion was accompanied by a diminished number of IS256 copies (Fig. 1C and Table 1). We made similar observations in a recent *in vitro* study using another ST27/ST2-related strain, called *S. epidermidis* 307 (10). The strain was isolated from a leukemia patient in Germany in 1994 and had PFGE restriction patterns that were very similar to those seen in isolates obtained from our current patient, demonstrating a close genetic relationship (Fig. 1B) (isolate 307). Interestingly, in *S. epidermidis* 307, the deletions of the *ica* genes were correlated with IS256 activity (M. Eckart and W. Ziebuhr, unpublished data). These data and the differences observed in the IS256-specific hybridization patterns among the patient's isolates lead us to speculate that similar IS256-mediated mechanisms may also occur *in vivo*.

This report, together with previously published data, demonstrates that specific *S. epidermidis* clonal lineages may pose a risk to vulnerable patients. The identification of strains belonging to these clonal lineages requires specific attention because, due to their genetic and phenotypic variability, these strains can easily be misidentified as contaminants. Positive blood cultures with CoNS are generally challenging for clinicians, and usually, antibiotic treatment is initiated only when more than one bottle is positive and the patient has a central venous catheter or indwelling foreign material, e.g., an artificial heart valve. ST27/ST2 isolates further complicate this difficult decision-making process, as phenotypically mixed cultures of CoNS are often not regarded as true pathogens, resulting in inappropriate or even no treatment. Given the virulence of these clones, this misinterpretation may put patients at a significant risk. Notably, the biofilm-forming capacity of ST27/ST2 isolates is a serious problem that requires specific treatment efforts. Thus, our patient had a central venous line which was changed repeatedly. However, in spite of an adequate management, blood cultures remained positive. Only upon addition of rifampin, an antibiotic known to act against biofilm-forming staphylococci (17, 22), did blood cultures eventually turn negative, indicating a critical role for biofilm formation in the persistence of the infection. The worldwide occurrence of ST27/ST2 strains in hospital settings suggests that the pathogenic potential of specific *S. epidermidis* clonal lineages should be reconsidered. The timely and correct identification of these

FIG. 1. (A) Biofilm-forming capacity of the *S. epidermidis* isolates (#1 to #12) on polystyrene tissue culture plates. *S. epidermidis* RP62A and *Staphylococcus carnosus* TM300 represent positive and negative controls, respectively. (B) PFGE of SmaI-restricted genomic DNA. #1 to #12, *S. epidermidis* patient isolates; 307, *S. epidermidis* strain 307 (see Discussion for details). Arrows indicate variants in which a DNA fragment of ~100 kb in size is lacking. (C) IS256-specific Southern hybridization of EcoRI-restricted chromosomal DNA. #1 to #12, *S. epidermidis* patient isolates.

clones may prompt adequate interventions and improve patient management. Systematic surveillance programs may help to closely monitor and prevent the further spread of these strains, thereby lowering the risk and costs of health care-associated infections in the future.

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