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The Bacterial Insertion Sequence Element IS*256* Occurs Preferentially in Nosocomial *Staphylococcus epidermidis* Isolates: Association with Biofilm Formation and Resistance to Aminoglycosides

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Staphylococcus epidermidis **is a normal constituent of the healthy human microflora, but it is also the most common cause of nosocomial infections associated with the use of indwelling medical devices. Isolates from device-associated infections are known for their pronounced phenotypic and genetic variability, and in this study we searched for factors that might contribute to this flexibility. We show that mutator phenotypes, which exhibit elevated spontaneous mutation rates, are rare among both pathogenic and commensal *S. epidermidis* strains. However, the study revealed that, in contrast to those of commensal strains, the genomes of clinical *S. epidermidis* strains carry multiple copies of the insertion sequence IS*256*, while other typical staphylococcal insertion sequences, such as IS*257* and IS*1272*, are distributed equally among saprophytic and clinical isolates. Moreover, detection of IS*256* was found to be associated with biofilm formation and the presence of the *icaADBC* operon as well as with gentamicin and oxacillin resistance in the clinical strains. The data suggest that IS*256* is a characteristic element in the genome of multiresistant nosocomial *S. epidermidis* isolates that might be involved in the flexibility and adaptation of the genome in clinical isolates.**

Staphylococcus epidermidis is a normal constituent of the healthy human skin and mucosal microflora. In recent decades, however, the bacterium has emerged as a nosocomial multiresistant pathogen and is now the most common cause of device-associated infections. Little is known of the factors that have contributed to this development, but the increasing number of immunocompromised patients, the use of indwelling medical devices, and a high selective pressure by antibiotics offer bacteria a novel ecological niche. It is unclear why just staphylococci were able to occupy this niche and by which factors pathogenic *S. epidermidis* differ from their commensal counterparts. In recent years, it has been shown that the ability to form biofilms on medical devices is a characteristic feature of nosocomial *S. epidermidis* isolates. Moreover, clinical *S. epidermidis* isolates exhibit an extraordinarily high phenotypic and genotypic flexibility. Thus, variants of the same parent strain can differ in terms of colony morphology, growth rate, hemolysis, biofilm formation, and antibiotic susceptibility (4, 7). The molecular mechanisms involved in this phenomenon are poorly understood, but it is assumed that the generation of phenotypic and genotypic variants is an evolutionary advantage that helps staphylococci to adapt to changing environmental conditions. The purpose of this study was therefore to search for genetic factors and mechanisms in clinical *S. epidermidis* that might contribute to this process. Previous studies have shown that staphylococcal biofilm formation is a highly variable factor which is influenced by both regulatory processes and genetic mechanisms such as phase variations, mutations, and chromosomal rearrangements (5, 10, 26, 32–34). The observation that some of these genetic processes are mediated by the action of insertion sequence (IS) elements prompted us to investigate the distribution of common staphylococcal IS elements among *S. epidermidis* strains of clinical and commensal origin. Moreover, we analyzed the relationship between IS presence, antibiotic resistance, and biofilm formation as well as the spontaneous mutation rate in this important nosocomial pathogen.

Bacterial strains. In this study, a total of 230 *S. epidermidis* strains, 139 of commensal origin and 91 clinical isolates (53 blood culture isolates and 38 isolates from urinary tract infections), were analyzed. Commensal strains were obtained by swabbing of the anterior nares of randomly selected outpatients who attended medical practitioners in the southwestern area of Germany. Patients with a hospitalization record or any other contact with a medical facility during a period of 3 months were excluded from the study. Blood culture isolates were recovered from intravenous catheter-related septicemia, and nosocomial urinary tract isolates were isolated from hospitalized patients suffering from catheter-associated urinary tract infections. Species diagnosis was verified by biochemical characterization using the API-20-Staph (bioMérieux, Marcy l'Etoile, France) system. All strains were tested for oxacillin resistance by growth on Mueller-Hinton agar supplemented with 3% sodium chloride and 6 μg of oxacillin/ml after a prolonged incubation period of 2 days at 30°C. There was a significant difference in terms of oxacillin resistance between clinical and saprophytic isolates ($P < 0.001$). Forty-four of 53 strains (83%) among the blood culture isolates and 5 of 38 strains (13%) among the urinary tract isolates were found to be resistant to oxacillin. Only 4 of the 139 commensal strains (3%)

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exhibited resistance to this β-lactam antibiotic (see Fig. 2). *mecA*-specific PCR confirmed the presence of the resistance-mediating *mecA* gene in all oxacillin-resistant isolates, while susceptible strains lacked this genetic information (data not shown).

Detection of IS*256*, IS*257*, and IS*1272*. In this study, we wanted to answer the question of whether pathogenic and nonpathogenic *S. epidermidis* differ with respect to the presence of IS elements in their genomes. We investigated the distribution of three typical IS elements which have been described previously as components of staphylococcal genomes, i.e., IS*256*, IS*257*, and IS*1272*. IS*256* was initially described as the flanking region of the composite aminoglycoside resistance-mediating transposon Tn*4001* (2). But the element also occurs in multiple, independent copies in the genomes of staphylococci and enterococci (9, 27). In previous studies, it was shown that IS*256* can be involved in phase variation of biofilm formation in *S. epidermidis* (3, 34). IS*257* is associated with the trimethoprim resistance-mediating transposon Tn*4003* and numerous other resistance genes and plasmids in staphylococci (e.g., cadmium resistance) (6). Isoforms of the element are also detectable on the SCCmec element in *S. aureus* and *S. epidermidis* (17). IS*1272* is detectable in many staphylococcal species and is prevalent in multiresistant clinical isolates (1, 18). IS elements were detected by IS-specific PCRs and Southern blotting. Amplification of IS*256* was performed using primers 5′-TGAAAAGCGAAGAGATTCAAAGC-3′ and 5′-ATGTAGGTCCATAAGAACGGC-3′ (GenBank accession no. of the published sequence, M18086). An IS*257*-specific gene probe was generated by combining primers 5′-GCTAATTTCGTGGCATGGCG-3′ and 5′-GTTATCACTGTAGCCGTTGG-3′ (accession no. X53952). *S. epidermidis* RP62A chromosomal DNA was used as a template. IS*1272* was amplified with primers 5′-GCTCGTTGAGCTACTTTTC-3′ and 5′-CCTAGAGAAATAGCCAGTAAATG-3′ (accession no. U35635) using *S. haemolyticus* 206 chromosomal DNA as a template. The gene probes were checked by nucleotide sequencing before they were applied for Southern hybridizations.

Figure 1 illustrates the distribution of IS*256*, IS*257*, and IS*1272* among the commensal and clinical strains. Interestingly, IS*256* was prevalent in 46 of 53 (87%) blood culture isolates and 18 of 38 (47%) urinary tract isolates but was prevalent in only 6 of 139 (4%) saprophytic strains. Statistical analysis using the chi-square test revealed significant differences in the distribution of IS*256* in blood culture isolates ($P < 0.001$) and urinary tract strains ($P < 0.001$) compared with that of the commensal strains. In contrast, no significant differences were recorded for the distribution of IS*257* and IS*1272* among clinical and saprophytic strains, respectively. Here, 47 of 53 (87%) blood culture isolates, 33 of 38 (87%) urinary tract isolates, and 124 of 139 (89%) saprophytic strains carried IS*257* copies. Similar results were obtained with respect to IS*1272*, which was detectable in 49 of 53 (92%) blood culture isolates, 37 of 38 (97%) urinary tract isolates, and 136 of 139 (97%) saprophytic strains. From these data, we conclude that IS*256* represents a specific element which is more likely to occur in clinical *S. epidermidis* strains than in commensal isolates.

Resistance towards gentamicin and detection of Tn*4001* and free IS*256* copies. Normally, IS*256* is part of the composite transposon Tn*4001*, which mediates gentamicin resistance by the bifunctional aminoglycoside-modifying enzyme AAC(6′)-APH(2″). To investigate whether or not the IS*256*-positive strains carry Tn*4001*, the isolates were tested for gentamicin resistance by the agar diffusion method according to the Deutsche Industrienorm 58940 guidelines and by an *aac(6′)-aph(2″)*-specific PCR using the primers 5′-GTATTAGAATTTTATGGTGG-3′ and 5′-CCATACATTCTTAATATATC-3′ under the following conditions: 1 min at 95°C, 1 min at 44°C, and 1.5 min at 68°C for 30 cycles. The resulting 1,184-bp PCR fragment was visualized by agarose gel electrophoreses and ethidium bromide staining. Resistance towards gentamicin was recorded in 46 of the 53 (87%) blood culture isolates, 18 of the 38 (45%) urinary tract isolates, and 6 of the 139 (4%) commensal strains (Fig. 2). Remarkably, 48 of 70 (68%) of the gentamicin-resistant strains, i.e., 41 of 44 (93%) blood culture isolates, 5 of 5 (100%) urinary tract isolates, and 2 of 4 (50%) oxacillin-resistant commensal strains were concomitantly resistant to oxacillin. In all gentamicin-resistant strains, the *aac(6′)-aph(2″)* gene was detectable by PCR, which implicates Tn*4001* in mediation of the aminoglycoside-resistant phenotype in the

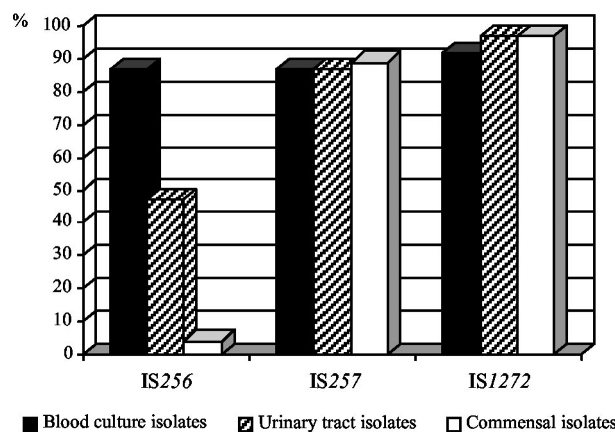

FIG. 1. Distribution of IS*256*, IS*257*, and IS*1272* among 230 *S. epidermidis* isolates from different origins. The study comprised 53 isolates recovered from blood cultures, 38 isolates obtained from catheter-related urinary tract infections, and 139 commensal strains obtained from nasal swabs of healthy volunteers.

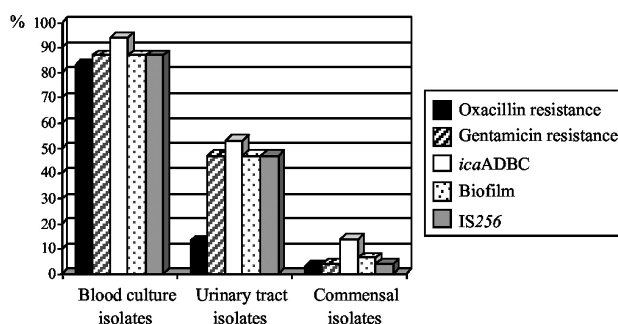

FIG. 2. Antibiotic resistance, biofilm formation, detection of the *icaADBC* operon, and IS*256* presence in clinical and commensal *S. epidermidis* strains.

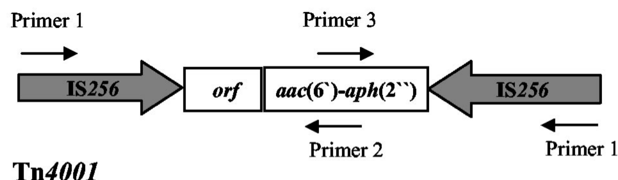

FIG. 3. Scheme of Tn*4001* and position of primers used for the detection of the transposon. (Please note that the image is not drawn to scale.)

IS*256*-positive strains. Interestingly, in a recent study on other coagulase-negative staphylococci, Tn*4001*-like elements were identified in which the IS*256* copies at the ends of the transposon were largely truncated and replaced by IS*257* (19). To search for such truncated Tn*4001* forms, we analyzed the 70 gentamicin-resistant strains by PCR using primers that cover the IS*256* and the *aac(6')-aph(2'')* genes (Fig. 3). When the entire Tn*4001* transposon is present, PCRs combining primer 1 (5′-TGAAAAGCGA AGAGATTCAAAGC-3′) and primer 2 (5′-CTAAACCGTGCATTTGTCTTA-3′) result in a DNA fragment of approximately 2.5 kb in size, while a PCR using primer 1 and primer 3 (5′-TTTAAGACAAATGCACGGTTT AG-3′) will amplify a 1.7-kb fragment. In 63 of 70 (90%) gentamicin-resistant isolates, both the 2.5- and 1.7-kb fragments were detectable, indicating the presence of the entire Tn*4001* transposon in these strains. Interestingly, in seven strains (two from blood cultures, four from urinary tract infections, and one from commensal isolates) no PCR products or smaller fragments were amplified, suggesting that these strains might harbor similar truncated Tn*4001* forms, as described recently by Lange et al. (19) (data not shown). However, more-detailed studies are necessary to support this hypothesis.

Southern hybridization of *Eco*RI-restricted chromosomal DNA with an IS*256*-specific gene probe revealed in 76% (53 of 70) of the *aac(6')-aph(2'')*-positive strains additional IS*256*-specific fragments which were unrelated to Tn*4001*, suggesting the existence of multiple free IS*256* copies of the element in these genomes (Fig. 4). The number of IS*256*-hybridizing fragments ranged from 1 (Tn*4001*-associated) up to 11. Recently, it has been shown that IS*256* transposes by a mechanism which involves circularization of the element and tandem dimer formation at the Tn*4001* ends (21, 24). Although the molecular mechanism of IS*256* transposition is not yet fully understood, it is tempting to speculate that it involves replication of the element. The two flanking IS*256* copies at the ends of Tn*4001* might therefore represent the origin of the multiple copies in *S. epidermidis* genomes.

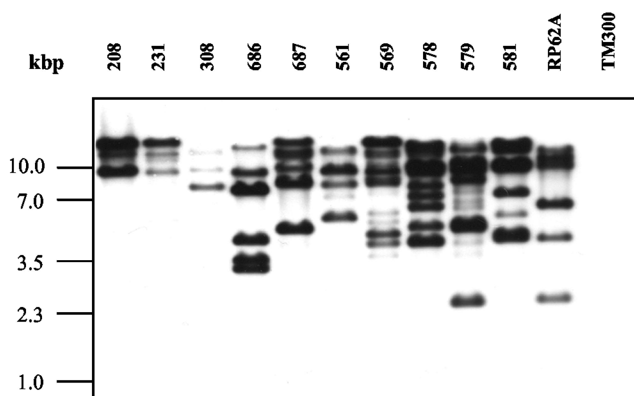

FIG. 4. Detection of multiple IS*256* copies by IS*256*-specific Southern hybridization of *Eco*RI-digested chromosomal DNA of different gentamicin-resistant *S. epidermidis* strains (208 to 581). *S. epidermidis* RP62A and *S. carnosus* TM300 were used as positive and negative controls, respectively.

***icaADBC* detection, biofilm formation, and IS*256*.** Previous studies have shown that IS*256* can influence the expression of biofilm formation in *S. epidermidis*. Staphylococcal biofilm formation is mainly mediated by the expression of the *icaADBC* operon, which encodes enzymes for the production of the polysaccharide intercellular adhesin PIA (16, 22). PIA expression undergoes phase variation, and in a substantial part of the variants this process is mediated by the alternating insertion and excision of IS*256* in an insertion hot spot of the *ica* operon (34). Moreover, the element seems to be involved in large rearrangements of the *S. epidermidis* genome which also affect biofilm formation and aminoglycoside resistance expression (32). To investigate a possible association between the *icaADBC* operon, biofilm formation, and IS*256*, all strains were tested for the presence of the *icaADBC* operon and biofilm formation as described previously (3, 33). The *ica* operon was detected in 94% (50 of 53) of the blood culture isolates and 53% (20 of 38) of the urinary tract isolates but in only 14% (20 of 139) of the saprophytic strains (Fig. 2). Statistical analysis of the data revealed that the *ica* operon is significantly more prevalent in strains from clinical origin than in commensal isolates ($P < 0.001$). After growth in tryptic soy broth supplemented with 3% sodium chloride, 47 of the 53 (87%) blood culture isolates, 18 of the 38 (47%) urinary tract strains, and 9 of the 139 (6.5%) commensal strains formed a detectable biofilm on polystyrene tissue culture plates (Fig. 2). A significant correlation was found between *icaADBC* presence and biofilm expression in the clinical strains ($P < 0.01$). Thus, 47 of the 50 (94%) *ica*-positive blood culture strains, 16 of the 20 (80%) *ica*-positive urinary tract isolates, and 7 of the 20 (35%) *ica*-positive commensal strains formed a visible biofilm on polystyrene tissue culture plates. Among the blood culture isolates, 3 isolates remained biofilm negative even though they carried the entire *icaADBC* operon, which was also the case with 4 urinary tract isolates and 13 *ica*-positive commensal strains. Among the commensal and urinary tract isolates, two strains were identified which exhibited a biofilm without the presence of the *icaADBC* operon, indicating that other factors might be involved in *S. epidermidis* biofilm formation as well. The data confirmed previous results from different studies indicating that biofilm formation and *icaADBC* presence are highly discriminating factors between clinical and commensal *S. epidermidis* (11, 12, 33). Interestingly, we observed that the majority of clinical strains carrying the *icaADBC* operon concomitantly harbored IS*256* in their genomes ($P \leq 0.05$). Thus, among the 50 *ica*-positive blood culture isolates, 88% (44 of 50) concomitantly carried IS*256*. A similar situation was observed with the urinary tract isolates. Here, we found among the 20 *ica*-positive strains 17 (85%) which were IS*256* positive. But only 3 of the 20 *ica*-positive

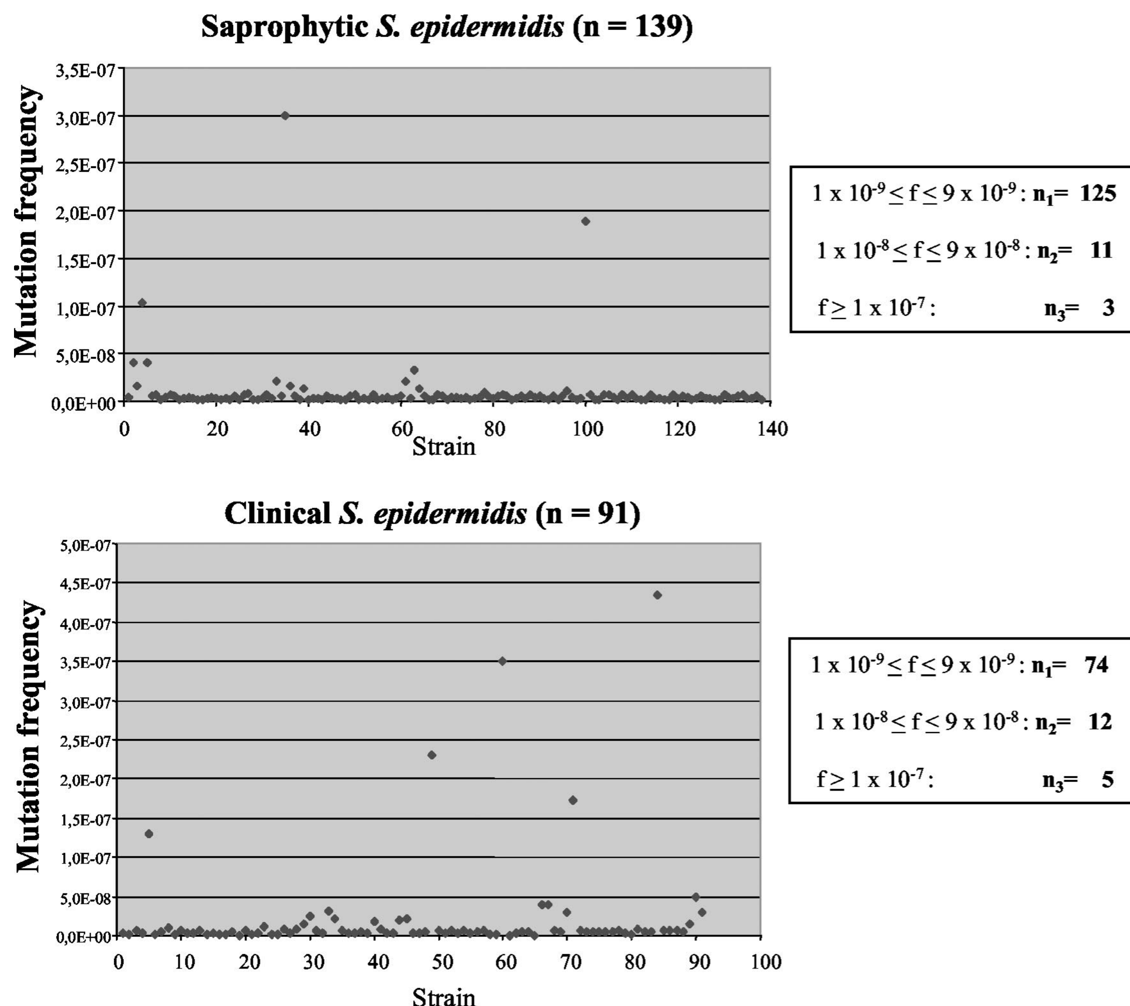

FIG. 5. Spontaneous mutation frequencies per cell and generation of the commensal and clinical *S. epidermidis* strains toward rifampin resistance.

commensal strains (15%) harbored IS*256* in their genomes (Fig. 2).

Determination of the spontaneous mutation rate of commensal and clinical *S. epidermidis*. The contribution of an elevated mutation rate to the virulence and adaptation of pathogens has been intensively and controversially discussed during recent years (8, 14, 15). Mutator phenotypes are supposed to have adaptive advantages and play a role in the pathogenesis of bacterial infections as well as in the development of antibiotic resistance (20, 23). Recently, hypermutable strains were identified in macrolide-resistant *S. aureus* isolates from cystic fibrosis patients (25). Moreover, defects in mismatch repair systems which result in an elevated mutation rate have been shown to contribute to the development of vancomycin resistance in *S. aureus* (29). Therefore, we wanted to investigate whether or not mutator phenotypes were detectable among our *S. epidermidis* isolates. Moreover, we wanted to elucidate possible differences in the mutation rates between the antibiotic-susceptible commensal strains and the multiresistant clinical strains. For this purpose, we determined the spontaneous mutation rate in the *rpoB* gene, which confers resistance to rifampin (23). The mutation rate per cell and generation was calculated as described previously (13, 34). Thus, a single bacterial colony was diluted in 45 ml of phosphate-buffered saline. One hundred microliters of this suspension was plated on agar plates to determine the inoculum size, and another 100-μl aliquot was used to inoculate 50 ml of Luria-Bertani broth, which was grown at 37°C to an optical density at 600 nm of 1.3 to 1.5. From this bacterial culture, 100-μl aliquots and appropriate dilutions were spread on Mueller-Hinton agar plates containing 10 μg of rifampin/ml. CFU were counted after a 24-h incubation at 37°C. The mutation frequency (P) to rifampin resistance per cell and generation was calculated using the formula

$$P = 1 - \sqrt[n]{1 - x}$$

where n is the number of generations and x is the number of rifampin-resistant colonies/total number of plated colonies. The number of generations (n) was determined using the equation

$$n = \frac{\lg N - \lg N_0}{\lg 2}$$

where N is the total number of bacterial cells in the culture and N_0 is the number of bacterial cells in the inoculum. Figure 5 indicates that the average mutation rate in the majority of the isolates is relatively low (approximately 10^{-9}). Of the 230 strains tested, 23 (10%) exhibited slightly elevated mutation rates of 10^{-8}, and only 8 strains (3.5%) had a spontaneous frequency greater than 10^{-7}. No difference was detectable between commensal and clinical strains with respect to the mutation rate. Moreover, we found no correlation between the slightly higher mutation rate and the presence of IS*256* in these strains. The data demonstrate that in both pathogenic, multiresistant *S. epidermidis* strains and nonpathogenic, antibiotic-susceptible *S. epidermidis* strains mutator phenotypes are rare. These findings suggest that in *S. epidermidis* strains from device-associated infections, point mutations might play a minor role in the pathogenesis of these infections.

Conclusions. *Staphylococcus epidermidis* is now a common pathogen which has been successfully established in the hospital environment. The majority of nosocomial *S. epidermidis* infections are catheter-related bloodstream infections, but the organism has also now emerged as a cause of urinary tract infections, preferentially in elderly, hospitalized patients carrying indwelling urinary tract catheters (28, 30, 31). Because staphylococci are also natural inhabitants of the skin, it is often difficult to decide whether an isolate represents the causative agent of an infection or an unspecific contamination of the specimen. Since this is specifically true for urinary tract infections, we also included in this study a group of nosocomial *S. epidermidis* isolates obtained from proven catheter-associated urinary tract infections from hospitalized patients. The data indicate that, in addition to the known differences in terms of biofilm formation and *icaADBC* presence, other factors exist which seem to be characteristic for pathogenic *S. epidermidis* isolates, from both line-associated septicemia and catheter-related urinary tract infections. Thus, a high proportion of the clinical isolates is resistant to oxacillin and gentamicin, and multiresistance in *S. epidermidis* is often accompanied by the presence of the biofilm-mediating *icaADBC* operon, which is expressed in the majority of the strains. The existence of *ica*-positive and *ica*-negative *S. epidermidis* strains raises the question of the origin of the biofilm-mediating *icaADBC* operon. It is conceivable that *ica*-positive *S. epidermidis* represent a single clone which acquired this genetic information and from which all biofilm-forming strains have evolved. Another possibility is the spread of the genes by horizontal gene transfer into different genetic backgrounds, and finally, it is possible that the *ica*-negative *S. epidermidis* represent deletion mutants in which the *ica* genes got lost. So far, there is no evidence to support any of these hypotheses, and more experimental work is needed to answer this important question in the future.

In contrast to other pathogens, where elevated mutation rates were proven to play a role in pathogenesis (20, 23), our study revealed no clue for an involvement of mutator phenotypes in device-associated *S. epidermidis* infections. However, the strains differed significantly with respect to the occurrence of IS elements in their genomes. Specifically, the presence of IS*256* seems to be a feature of pathogenic *S. epidermidis* strains. In our strain collection, IS*256* was exclusively associated with Tn*4001*, but the element also occurred independently in multiple free copies in these strains. In general, mobile genetic elements, such as insertion sequences, transposons, phages, and genomic islands, are common components of microbial genomes. Together with point mutations, homologous recombination, and horizontal gene transfer, mobile DNA elements are driving forces for the generation of novel genetic and phenotypic variants. It is tempting to speculate that the presence of multiple IS*256* copies might play a role in the flexibility of the genome of multiresistant, biofilm-forming *S. epidermidis* isolates. This model could represent an advantage in the rapid adaptation of the bacterium to changing environmental conditions, and the underlying genetic mechanisms and effects therefore merit further detailed analyses.

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