JOURNAL OF CLINICAL MICROBIOLOGY, June 2001, p. 2151–2156 Vol. 39 No. 6
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.6.2151–2156.2001

Presence of *icaA* and *icaD* Genes and Slime Production in a Collection of Staphylococcal Strains from Catheter-Associated Infections

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Received 15 May 2000/Returned for modification 25 July 2000/Accepted 23 February 2001

Both *Staphylococcus epidermidis* and *Staphylococcus aureus* are important causes of infections associated with catheters and other medical devices. It has recently been shown that not only *S. epidermidis* but also *S. aureus* can produce slime and carries the *ica* operon responsible for slime production. In the operon, coexpression of *icaA* and *icaD* is required for full slime synthesis. In this study, the presence of *icaA* and *icaD* was determined in a collection of 91 staphylococcal (68 *S. epidermidis* and 23 *S. aureus*) strains from intravenous catheter-associated infections, in 10 strains from the skin and mucosa of healthy volunteers, and in two reference strains by a PCR method. Slime-forming ability was tested on Congo red agar plates; 49% of *S. epidermidis* strains from catheters and, surprisingly, 61% of *S. aureus* strains were *icaA* and *icaD* positive and slime forming. All the saprophytic strains turned out to be negative for both *icaA* and *icaD* and also non-slime forming. Two *S. aureus* and one *S. epidermidis* strain from catheters, detected as *icaA* and *icaD* positive by PCR analysis and as slime forming (black colonies) at 24 h on Congo red agar, at 48 h exhibited tiny red spikes at the center of black colonies. The onset of these variants could not be ascribed to a mutagenic potential of Congo red, which, in the Ames test, was devoid of mutagenicity. PCR analysis showed that these red variants were negative for both *icaA* and *icaD* and even lacking the entire *icaADBC* operon. The data reported indicate an important role of *ica* genes as a virulence marker in staphylococcal infections from intravenous catheters.

Staphylococcus epidermidis is a saprophyte which is part of the normal mucosa and skin microflora. In recent years, however, *S. epidermidis* emerged, together with *Staphylococcus aureus*, as a frequent etiologic agent of infections associated with catheters and other indwelling medical devices. As they possess little intrinsic pathogenic power, staphylococci are usually regarded as opportunistic agents (16, 18). Over the last few years, several studies have been done to elucidate the structures and pathogenetic mechanisms by which staphylococci are able to cause severe and irreducible infections associated with biomaterials (4, 13, 22). As far as *S. epidermidis* is concerned, polysaccharide slime (also called biofilm) seems to be the most important factor by which it adheres to and colonizes artificial materials (31). As for *S. aureus*, it was well known, until now, for its ability to express molecules which recognize host matrix proteins (8, 10, 23, 32). It has recently been shown that *S. aureus* as well as *S. epidermidis* is capable of forming slime (2, 5, 9, 21, 23).

Recently, the genetic control of slime production has begun to be elucidated (17), first in *S. epidermidis* and then in *S. aureus* (9, 21). Synthesis of the capsular polysaccharide is mediated by the *ica* operon. Upon activation of this operon, a polysaccharide intercellular adhesin is synthesized. This supports cell-to-cell bacterial contacts by means of a multilayered biofilm. The polysaccharide intercellular adhesin is composed of linear β-1,6-linked glucosaminylglycans. It is synthesized in vitro from UDP-*N*-acetylglucosamine by the enzyme *N*-acetylglucosaminyltranferase, which is encoded by the intercellular adhesion (*ica*) locus, in particular by the *icaA* gene. Sole expression of *icaA* induces only low enzymatic activity, but coexpression of *icaA* with *icaD* leads to a significant increase in activity and is related to phenotypic expression of the capsular polysaccharide (17).

The aim of this study was to determine the occurrence of the *icaA* and *icaD* genes for slime production in a collection of staphylococcal clinical isolates by a simple, rapid, and reliable PCR method previously developed in our laboratory. The search for *ica* genes was carried out in two *S. epidermidis* reference strains, 68 *S. epidermidis* isolates from intravenous catheter-associated infections, 23 *S. aureus* isolates from catheter-associated infections, and 10 *S. epidermidis* strains from the skin and mucosa of healthy volunteers. Slime-forming ability was evaluated by the Congo red agar (CRA) plate test.

MATERIALS AND METHODS

Bacterial strains. Two *S. epidermidis* reference strains were used, the well-known slime-producing strain ATCC 35984 (RP62A) and the non-slime-producing strain ATCC 12228.

The present study focused on 91 staphylococcal isolates from intravenous catheter-associated infections. These included 68 strains of *S. epidermidis* and 23 strains of *S. aureus*. A further 10 strains of *S. epidermidis* isolated from the skin or mucosa of healthy volunteers were also investigated. All isolates were characterized by classic microbiological methods. In particular, the staphylococcal species were identified by the Api-Staph test (Biomérieux, Lyon, France), a biochemical identification kit, and the coagulase test.

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Phenotypic characterization of slime-producing ability. Production of slime from all strains was studied by cultivation of the strains on CRA (15). CRA plates (0.8 g of CRA [Sigma] and 36 g of saccharose [Sigma] to 1 liter of brain heart infusion agar [Oxoid, Basingstoke, Hampshire, England]) were incubated for 24 h at 37°C and subsequently overnight at room temperature. On CRA, slime-producing strains form black colonies, whereas nonproducing strains develop red colonies. In some cases, when pink subcolonies emerged at 48 h in the center of the black colonies, small pink or black samples were picked from both pink and black areas and subcultured again for 24 h on CRA to obtain pure isolates of slime-negative and slime-positive variants.

Ames test. In order to test if Congo red had mutagenic potential in the CRA plate test, the Ames test was carried out according to the method described by Ames et al. (1) and revised by Maron and Ames (20). Two strains of *Salmonella enterica* (TA100 and TA1538) were used. Congo red was added to the top agar at a final concentration equal to that used in the CRA test (0.8 g/liter). The experiments were carried out in the absence and in the presence of the rat liver microsomal fraction S9 (Moltox, Annapolis, Md.) at a concentration of 20 μl/plate.

Negative and positive controls for TA100 and TA1538 were run in parallel. The former was intended to evaluate the number of spontaneous revertants, the latter to evaluate the response of the *S. enterica* strains toward a known mutagen (2-aminofluorene at a concentration of 10 μg/plate). Counting of revertant colonies was carried out after a 48-h incubation at 37°C.

Strain storage. A single colony of each bacterial strain was seeded in 8 ml of Trypticase soy broth (TSB). After incubation for 24 h at 37°C, the broth culture was fractioned into 1-ml aliquots, which were stored at −80°C.

Solutions for bacterial lysates. For lysostaphin (Sigma), a stock solution (1 mg/ml in H_2O) was stored in small aliquots at −20°C. Before use, the enzyme was diluted 1:10 with H_2O to give a 100 μg/ml concentration. For proteinase K (Sigma), a stock solution (1 mg/ml in H_2O) was stored in small aliquots at −20°C. Before use, the enzyme was diluted 1:10 with H_2O to give a 100 μg/ml concentration. The buffer was 0.1 M Tris-HCl (pH 7.5).

Bacterial DNA extraction. Bacteria were harvested by centrifuging 100 μl of each broth culture. Cells were resuspended in 45 μl of H_2O, 5 μl of lysostaphin solution was added, and samples were incubated at 37°C. After 10 min, 5 μl of proteinase K solution and 150 μl of 0.1 M Tris-HCl (pH 7.5) were added, and incubation proceeded for a further 10 min. Samples were then heated for 5 min at 100°C.

PCR method for amplification of *icaA* and *icaD* sequences. The sequences of *icaA* and *icaD* were taken from the GenBank sequence database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) (accession number for the *ica* operon, U43366).

Primers specific for *icaA* and *icaD* were picked on the gene sequences by the Primer3 program (National Institutes of Health National Human Genome Research Institute [http://www.genome.wi.mit.edu/genome_software/other/primer3.html]).

The primers were synthesized by M-Medical Genenco (Florence, Italy). For the detection of *icaA*, 5′-TCTCTTGCAGGAGCAATCAA was used as the forward primer (primer 1, corresponding to nucleotides 1337 to 1356), and 5′-TCAGGCACTAACATCCAGCA was used as the reverse primer (primer 2, corresponding to nucleotides 1505 to 1524). The two primers include a 188-bp region. For detection of *icaD*, 5′-ATGGTCAAGCCCAGACAGAG was used as the forward primer (primer 1, corresponding to nucleotides 1963 to 1982), and 5′-CGTGTTTTCAACATTTAATGCAA was used as the reverse primer (primer 2, corresponding to nucleotides 2138 to 2160). The two primers include a 198-bp region. PCR was performed in a DNA thermal cycler (UNO II Thermocycler; Biometra GmbH, Gottingen, Germany). The reaction was in a 25-μl volume containing the above-mentioned primers (1 μM each), together with 150 ng of the extracted DNA, 100 μM each of dATP, dCTP, dGTP, and dTTP, 1 U of *Taq* DNA polymerase, and buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 0.1% Triton X-100, and 2.5 mM $MgCl_2$). A thermal step program for both *icaA* and *icaD* was used, including the following parameters: incubation at 94°C for 5 min, followed by 50 cycles at 94°C for 30 s (denaturation), 55.5°C for 30 s (annealing), 72°C for 30 s (extension), and 72°C for 1 min after conclusion of the 50 cycles. After the first 30 cycles, a further 1 U of *Taq* DNA polymerase was added. After amplification, 10 μl of the PCR mixture was analyzed by agarose gel electrophoresis (2% agarose in Tris-borate-EDTA). Molecular weight marker kit VI (Boehringer Mannheim) was used.

PCR method for amplification of *ica* operon. The presence of the entire *ica* operon in slime-producing strains and in nonproducing red variants of both *S. epidermidis* and *S. aureus* was checked by amplifying a gene product of 2.7 kb, encompassing a region of the *icaADBC* locus, as described (21).

RESULTS

Detection of slime-producing phenotype of *S. epidermidis* and *S. aureus* strains by CRA plate test. Phenotypic production of slime by all strains under study was assessed by culture on CRA plates. As shown in Fig. 1, slime-producing strains appear as black colonies, and non-slime-producing strains appear as red colonies. *S. aureus* colonies on CRA were kept under observation for up to 72 h, since the onset of black coloration, indicating slime production, occurs earlier for *S. epidermidis* than for *S. aureus* (24 versus 48 to 72 h; unpublished data). Application of Christensen's method to *S. aureus* requires the addition of glucose for evident slime production to occur (2). The coloration delay found in the CRA method, which uses saccharose as a carbohydrate source, can thus be seen to correspond to the known glucose requirement of *S. aureus* in Christensen's method. This suggests that *S. aureus* and *S. epidermidis* follow different metabolic pathways in the formation of the *N*-acetylglucosamine slime precursor.

Among the clinical isolates, 33 of 68 (48.5%) *S. epidermidis* strains and 14 of 23 (60.8%) *S. aureus* strains turned out to be slime producing. All 10 saprophytic *S. epidermidis* strains from skin and mucosa were non-slime producing.

PCR detection of *icaA* and *icaD*. The PCR technique was applied to the 103 staphylococcal strains. As shown in Fig. 2, all strains which were positive for *icaA* were also positive for *icaD*. The slime-producing reference strain ATCC 35984 (RP62A) was found to be positive for both genes, giving a 188-bp band for the *icaA* gene and a 198-bp band for the *icaD* gene. The non-slime-producing *S. epidermidis* reference strain ATCC 12228 was negative for both genes. In the saprophytic strains of *S. epidermidis* from the skin or mucosa of healthy volunteers, neither band was found. Both bands were obtained in slime-producing clinical isolates of both *S. epidermidis* and *S. aureus*. Conversely, no band was obtained from non-slime-producing clinical isolates. After determining the sizes of standard bands with the molecular weight marker kit, the image analyzer system assigned the expected lengths to the bands obtained by amplification of the DNA extracted from slime-producing strains. Figure 2 shows the detection of *icaA* and *icaD* by PCR.

Phase variants of *S. epidermidis* and *S. aureus* clinical isolates. One *S. epidermidis* and two *S. aureus* clinical isolates, which at 24 h appeared as black colonies on CRA, at 48 h exhibited tiny pink or red spikes at the center of the black colonies (Table 1 and Fig. 1). Pure black and pure red colonies were obtained on subcultures of samples picked separately from black areas and pink or red spikes, respectively. PCR analysis of these subcolonies showed that the pink and red variants were negative for both *icaA* and *icaD*. Moreover, PCR amplification of a 2.7-kb region representing almost the entire *ica* locus clearly shows that in red variants, not only the *icaA* and *icaD* segments were lacking, but even the entire *ica* locus was absent (Fig. 3).

Lack of mutagenicity of Congo red. Congo red, when assayed at the concentration used in and under the conditions of the CRA test, was not mutagenic toward the two *S. enterica* strains tested in either the absence or presence of S9 microsomal extract. The results of the Ames test are shown in Table 2.

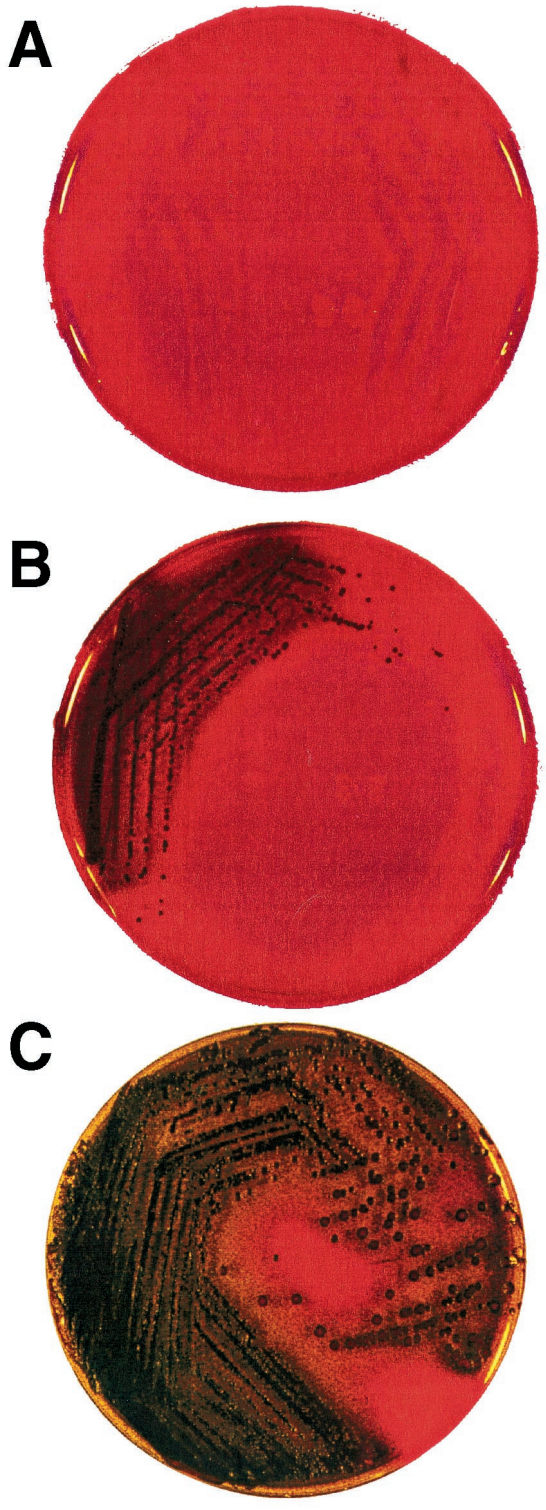

FIG. 1. CRA plate test. (A) Red colonies of the non-slime-producing *S. epidermidis* reference strain ATCC 12228. (B) Black colonies of the slime-producing *S. epidermidis* reference strain ATCC 35984 (RP62A). (C) Black colonies of *S. epidermidis* strain 13, which exhibited a red central zone at 48 h, indicating the onset of a non-slime-producing phase variant.

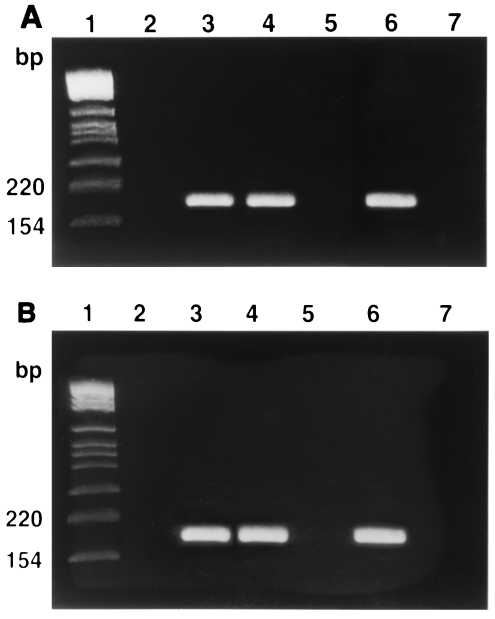

FIG. 2. PCR detection of *icaA* and *icaD* genes. (A) PCR results with primers for *icaA*. Lane 1, molecular size markers; lane 2, absence of band with DNA from non-slime-producing *S. epidermidis* reference strain ATCC 12228; lane 3, 188-bp band obtained with DNA from slime-producing *S. epidermidis* reference strain ATCC 35984 (RP62A); lane 4, 188-bp band obtained with DNA from slime-producing *S. epidermidis* strain 13; lane 5, absence of band with DNA from red variant of *S. epidermidis* strain 13; lane 6, 188-bp band obtained with DNA from slime-producing *S. aureus* strain 9; lane 7, absence of band with DNA from red variant of *S. aureus* strain 9; lane 8, negative control (DNA template absent). (B) PCR results with primers for *icaD*. Lane 1, molecular size markers; lane 2, absence of band with DNA from non-slime-producing *S. epidermidis* reference strain ATCC 12228; lane 3, 198-bp band obtained with DNA from slime-producing *S. epidermidis* reference strain ATCC 35984 (RP62A); lane 4, 198-bp band obtained with DNA from slime-producing *S. epidermidis* strain 13; lane 5, absence of band with DNA from red variant of *S. epidermidis* strain 13; lane 6, 198-bp band obtained with DNA from slime-producing *S. aureus* strain 9; lane 7, absence of band with DNA from red variant of *S. aureus* strain 9; lane 8, negative control (DNA template absent).

DISCUSSION

Bacterial adhesion has long been considered as a virulence factor contributing to infections associated with catheters and other indwelling medical devices. Interaction of bacteria with biomaterials has been suggested to have a crucial role in conditioning the progress of these severe nosocomial infections (14). The existence of different bacterial molecules of adhesion has been increasingly documented. The possibility was considered that, in the interaction with biomaterials, bacterial cells exhibited various mechanisms, according to environmental conditions and surface characteristics of both material and bacterium. Fletcher and Marshall (12) noted that, with proteolytic enzyme treatment, a *Pseudomonas* strain could be detached from polystyrene surfaces but not from glass ones, suggesting that different adhesion mechanisms are involved for different materials.

For staphylococcal species, two possible explanations of the ability to colonize artificial materials are the bacterial production of polysaccharide slime and the presence of adhesins for

TABLE 1. Association between slime production on CRA and presence of *icaA* and *icaD* in *Staphylococcus* strains isolates from catheter infections and from mucosa and skin of healthy volunteers

Strain	No. of isolates	Origin of infection	Colony color at:		Slime production	*icaA*	*icaD*
			24 h	48 h			
Reference							
ATCC 35984 (RP62A)			Black	Almost black	Strong	+	+
ATCC 12228			Red	Red	No	−	−
S. epidermidis catheter isolates							
1-9-14-20-22-55-60-P13-3561-7P-8P-22P-33P-35P-42AP-47P-49P-2ior-21	19	Catheter	Black	Black	Strong	+	+
13	1	Catheter	Black	Black[a]	Strong	+	+
15-B-465-948-38P-52P-54P-58P-7ior-16-28-43-5P	13	Catheter	Almost black	Black	Strong	+	+
4-5-7-8-10-22ior-35ior-21-24-61-948-930-0B49-20P-21P-32P	16	Catheter	Red	Red	No	−	−
78ior-48P-45P-50P-44P	5	Catheter	Red	Deep red	No	−	−
2-11-23-462-470-3P-17P-23P-30P-31P-34P-40P-41P-46P	14	Catheter	Deep red	Deep red	No	−	−
S. aureus catheter isolates							
3-7-13-14-19	4	Catheter	Black	Black	Strong	+	+
6	2	Catheter	Almost black	Black	Strong	+	+
9-15	2	Catheter	Black	Black[a]	Strong	+	+
2-4	1	Catheter	Bordeaux	Black	Strong	+	+
1-17-20-23	5	Catheter	Bordeaux	Almost black[b]	Strong	+	+
22	1	Catheter	Red	Deep red	No	−	−
10-12-16-21	4	Catheter	Deep red	Deep red	No	−	−
5-8-11-18	4	Catheter	Red	Red	No	−	−
S. epidermidis isolates from healthy volunteers							
798-799-800-801-802-803	6	Mucosa	Deep red	Deep red	No	−	−
901-902-903-904	4	Skin	Deep red	Deep red	No	−	−

[a] Colonies that were black at 24 h but which exhibited tiny pink or red central spikes at 48 h.
[b] *S. aureus* colonies kept under further observation for up to 72 h before turning black.

the host matrix proteins that, in vivo, are adsorbed onto the biomaterial surface (8, 10, 23).

Molecular techniques for detection of the gene sequences that encode the adhesion molecules could conveniently be applied in the study of prevalent adhesion mechanisms. From the clinical standpoint, elucidation of the main adhesive mechanisms in periprosthesis infections may help in developing preventive and therapeutic measures, such as antiadhesive coatings or antiadhesin drugs (3, 5, 6, 7, 27, 30).

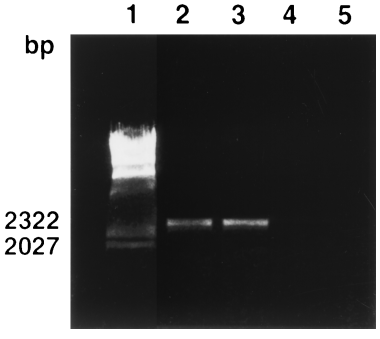

FIG. 3. PCR detection of *ica* locus in slime-producing staphylococcal clinical isolates and in their nonproducing red variants. Lane 1, molecular size markers (lambda *Hin*dIII digest [Sigma]); lane 2, 2.7-kb band obtained with DNA from slime producer *S. epidermidis* strain 13; lane 3, 2.7-kb band obtained with DNA from slime producer *S. aureus* strain 9; lane 4, absence of band with DNA from red variant of *S. epidermidis* strain 13; lane 5, absence of band with DNA from red variant of *S. aureus* strain 9.

We have previously described PCR methods for rapid identification of genes encoding the main microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (28) involved in prosthesis adhesion, such as the fibronectin binding protein genes (*fnbA* and *fnbB*) (25) and the collagen adhesin gene (*cna*) (24). Investigation of the presence and expression of *cna* in a collection of *S. aureus* strains from orthopedic periprosthesis infections has revealed that the slime-positive strains predominate over the *cna*-positive ones and that a striking association exists between these two adhesion mechanisms (23).

The data reported here indicate an important role of the *ica* locus as a virulence marker for clinically significant staphylococcal isolates. Its presence in a high percentage of clinical isolates and its association with the strains' ability to produce slime strongly suggest a role of *icaA* and *icaD* in the pathogenetic mechanisms of infection associated with catheters. This should allow routine diagnostic identification of particularly virulent staphylococcus strains. The method recognizes slime-

TABLE 2. Ames test for Congo red with two *S. enterica* test strains

Addition to agar	Mean no. of revertant colonies/plate ± SD (n = 3)			
	Strain TA100		Strain TA1538	
	S9 absent	S9 present	S9 absent	S9 present
Congo red	172 ± 7	123 ± 6	20 ± 3	19 ± 3
Negative control	165 ± 7	119 ± 8	21 ± 3	20 ± 3
Positive control	421 ± 18	313 ± 11	252 ± 25	252 ± 25

forming strains of both *S. epidermidis* and *S. aureus*, requires minimal amounts of DNA, and can thus be readily applied to biopsy material. In the present investigation, staphylococcal strains were retrieved from infected catheters, but in other cases, mainly orthopedic infections, either needle biopsy material or small fragments retrieved from the periprosthesis infected tissues proved to be suitable for PCR amplification. Biopsy material was seeded in TSB, and after incubation for 24 h at 37°C, bacteria were harvested by centrifugation, DNA was extracted, and *ica* genes were detected by PCR.

For *S. epidermidis*, the percentage of slime-producing strains (49%) is very close to that found by Muller et al. (46%) for catheter-related infections (26). Ziebuhr et al. (33) have instead reported that 87% of *S. epidermidis* clinical isolates from catheter infections are slime forming, but the strains were isolated from blood cultures and not directly from catheters, as in our present study. With regard to slime production by *S. aureus* in catheter-related infections, the data reported provide interesting confirmation of recent observations regarding slime-producing capacities (2, 5) and the presence of *ica* genes in *S. aureus* (17). Surprisingly, more slime-producing strains were recorded for *S. aureus* than for *S. epidermidis* (61 versus 49%). Thus, far from being peculiar to *S. epidermidis*, as was once thought, slime production appears to be at least as common in *S. aureus*. Difficulties in adapting in vitro methods designed for phenotypic study of these abilities in *S. epidermidis* (11, 15) to *S. aureus* may have hampered their recognition, as well as the lack, until now, of molecular tests for the detection of genes which control the synthesis of slime. The present results indicate that, among the clinical isolates from catheter infections, 49% of *S. epidermidis* and 61% of *S. aureus* are slime producing. These data must be considered reliable and not the outcome of a bias, since the entire catheter specimen is put in culture and the risk of selective isolation of more readily detachable, nonadherent, *ica*-negative variants can be ruled out. Since, however, adhesion is a prerequisite for the development of an infection, the slime-negative strains must possess other mechanisms which explain their infectious aptitude. The *ica*-negative, non-slime-producing isolates likely represent strains with alternate means of adhesion, such as MMSCRAMS, relevant in causing catheter-related infections. Actually, in a large collection of clinical isolates from catheter-associated infections, we have found that a large proportion of both *S. aureus* and *S. epidermidis* strains, either *ica* positive or *ica* negative, harbor the two genes for the fibronectin binding protein (*fnbA* and *fnbB*) (unpublished results). This finding reinforces the opinion that mechanisms beside slime production are responsible for bacterial adhesion.

In the present study, PCR analysis also showed that the red variants from three *icaA*- and *icaD*-positive, slime-forming isolates from catheters (two *S. aureus* and one *S. epidermidis*) were negative for both *icaA* and *icaD* and that, moreover, in these variants the entire *ica* locus seems to be lacking. These data suggest that the phenotypic change may be caused by a deletion of the *ica* operon rather than an insertion event which inactivates the *ica* genes. Ziebuhr et al. found that, in *S. epidermidis*, the insertion of a 1,332-bp sequence element, known as IS*256*, causes inactivation of *icaA* gene in non-slime-forming variants of slime-forming *S. epidermidis* strains (34). A single insertion point has been described in *icaA*. However, the *icaA* amplification region selected in this study (nucleotides 1337 to 1524) should be sufficiently distant from the insertion point of IS*256* in *icaA* (at 1708) to ensure that the PCR product is unaffected by this insertion phenomenon. Moreover, no insertion has been described in *icaD* (34). The present evidence, that in non-slime-producing red variants the *ica* locus is lacking, could also be explained by the possibility that rare slime-negative mutants, originally present in the slime-positive clinical isolates, emerge as red spikes upon passages and prolonged culture on CRA plates. Moreover, a direct mutagenic effect of Congo red must be ruled out, since, at the concentration employed in the plate test, the dye does not show any effect in the Ames test. This finding is in agreement with a previous observation by Reid et al. (29). These authors showed that Congo red was not mutagenic toward *S. enterica* TA1538 unless incubated overnight in the presence of rat intestinal bacteria and subsequently assayed in the presence of a postmitochondrial activating system.

In conclusion, we suggest that a study of the presence and expression of genes for adhesion molecules, such as the *ica* genes, may help in clarifying the relevance of the different adhesion mechanisms in the pathogenesis of infections associated with medical devices. It could also be of value in the development of new preventive and therapeutic measures.

ACKNOWLEDGMENTS

This work was supported by Italian Ministry of Health grant SVE 4-ICS/RF98-610 (Interaction between opportunistic bacteria and biomaterials).

We are grateful to Chiara Vescovini and Roberta Rambaldi for skilled assistance in preparing the manuscript. The technical assistance of Daniela Cavedagna and M. Elena Donati is also gratefully acknowledged. We are also grateful to Marina Cervellati for carrying out the Ames test.

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