Biofilm Formation on Intraocular Lenses by a Clinical Strain Encoding the *ica* Locus: A Scanning Electron Microscopy Study

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PURPOSE. To determine whether the *Staphylococcus epidermidis* strain carries the intercellular adhesion (*ica*) locus, which encodes production of adhesins mediating adherence to biomaterials and to study, with scanning electron microscopy, the morphologic features of this coagulase-negative *Staphylococcus* strain that adheres to intraocular lenses (IOLs).

METHODS. Polymerase chain reaction amplification was used to investigate whether the isolate under study (*S. epidermidis* clinical strain N890074) carries the *ica* locus. Sterile intraocular lenses (IOLs) were incubated in bacterial suspension either for 5 minutes or 1 hour. IOLs were then examined by scanning electron microscopy.

RESULTS. Polymerase chain reaction amplification revealed that *S. epidermidis* N890074 contained the *ica* locus. The bacteria appeared to be anchored to the surface of the lenses by several different means—particularly by leglike appendages and a slime layer—which probably came into play step by step.

CONCLUSIONS. For the first time in ophthalmology, to the authors' knowledge, photographs showing leglike appendages involved in the first phase of adhesion have been obtained. They also clearly visualize the slime layer containing the embedded bacteria. This study provides information about the nature and the genesis of these attachment processes. Adherence is known to be greater when the bacterial DNA contain the *ica* locus. Full knowledge of the pathogenesis of bacterial adhesion is necessary to gain a better understanding of IOL infection and endophthalmitis. (*Invest Ophthalmol Vis Sci.* 2003;44:4382–4387) DOI:10.1167/iovs.03-0185

Postoperative endophthalmitis is still one of the worst complications of intraocular lens (IOL) implantation. It remains difficult to anticipate and represents a therapeutic emergency because of its rapid evolution and poor prognosis. Today, although endophthalmitis may be cured with a minimum of aftereffects, there are too many cases that remain responsible for a definitive functional loss (50% of patients recover a visual acuity of no more than 20/400) or even for anatomic eye loss.[1] The exact mechanisms by which the bacterium *Staphylococcus epidermidis* causes endophthalmitis after cataract surgery are not completely known. Nevertheless, the binding of bacteria on IOLs during implantation is well known to represent a prominent factor in the pathogenesis of endophthalmitis and of pseudophakic chronic intraocular inflammations.[2–6] Colonization of IOLs by *S. epidermidis*, the germ most often involved,[6] eventually leads to production of a biofilm that may prevent antibiotics from gaining access to the microorganism and reduce the efficacy of host defenses.[7–13] Thus, it would be beneficial to decrease the incidence of endophthalmitis and its pathogenicity by reducing the adherence of bacteria to intraocular implants and thus inhibit biofilm production.

Biofilm formation is classically thought to be a two-step process,[12,14–16] but only rare studies have shown this by providing photographs, none of which were taken in ophthalmology to our knowledge. The two sequential steps required are adhesion of cells to a solid substrate followed by cell–cell adhesion.[16] The first phase is mediated by nonspecific physicochemical forces and/or the capsular polysaccharide/adhesin (PS/A) and/or several surface proteins. The second phase is mediated by the bacterial production of a polysaccharide glycocalyx (slime) on the IOL surface[5,16,17] containing another antigen called polysaccharide intercellular adhesin (PIA). A recent study has shown that the intercellular adhesion (*ica*) locus of *S. epidermidis* encodes production of both PS/A and PIA.[14]

The purpose of the present study was to describe and consequently to improve the understanding of the interactions between bacteria, IOLs, and biofilm formation. In fact, total knowledge of the pathogenesis of IOL infections is needed to overcome their development and to reach clinical efficacy. In this article, polymerase chain reaction (PCR) amplification was used to ascertain whether the strain under study (already used in one of our previous studies[2]) carries the *ica* locus, which is known to encode production of *S. epidermidis* antigens, mediating adhesion to biomaterials and between the bacterial cells. In addition, we report the results of a scanning electron microscopy (SEM) study of this coagulase-negative *Staphylococcus* strain that adheres to IOLs.

MATERIALS AND METHODS

Intraocular Lenses

New sterile 575 SM silicone IOLs with polymethylmethacrylate (PMMA) haptics, provided by Corneal (Annecy, France), were used throughout the study. All of them had identical optical diameters (5.75 mm) and dioptric power (22 D).

Bacterial Strain

The microbiology department of Edouard Herriot Hospital (Staphylococci National Reference Centre, Lyon, France) provided a clinical isolate of *S. epidermidis* (N890074). This strain was isolated from
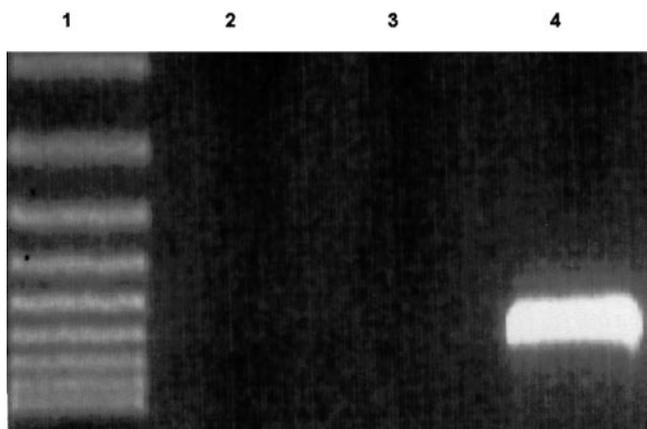
FIGURE 1. Agarose gel electrophoresis of polymerase chain reaction amplification of *icaA-B* genes. *Lane 1*: 100-bp DNA ladder; *lane 2*: negative control (water); *lane 3*: *S. epidermidis* (American Type Culture Collection 14990); *lane 4*: *S. epidermidis* N890074. The bright band in *lane 4* represents the *icaA* and *icaB* genes.

infected cerebrospinal fluid due to a complication of a ventriculoperitoneal shunt in a child with hydrocephalus. N890074 was identified by colony and microscopic morphology, by the absence of coagulase activity in rabbit plasma (bioMérieux, Marcy l'Etoile, France), by the absence of production of a clumping factor (Staphyslide; bioMérieux), and by results obtained with a *Staphylococcus* gallery (ID32; bioMérieux). This isolate can produce a great amount of slime.[2] The bacterial concentration was spectrophotometrically adjusted to 10^8 colony-forming units (CFU) per milliliter in a sterile 0.08-M phosphate-buffered (pH 7.8) saline solution (PBS buffer). Complete lenses (including haptics) were incubated at 37°C in the bacterial suspension, with continuous shaking, either for 5 minutes or for 1 hour, before they were washed three times in a sterile 0.08-M PBS (pH 7.8) solution to remove unbound bacteria.

DNA Preparation, Amplification, and Sequencing

Genomic DNA was extracted from agar plate cultures, as previously described[18] and used as a template for PCR amplification with the primers icaAB-forward (5′-CATGGTCAA GCCCAGACA-3′) and icaAB-reverse (5′-CATAAATCGT TTCATCCATA TCATCAAAGT T-3′) (Eurogentec, Liége, Belgium) which were chosen to encompass a fragment
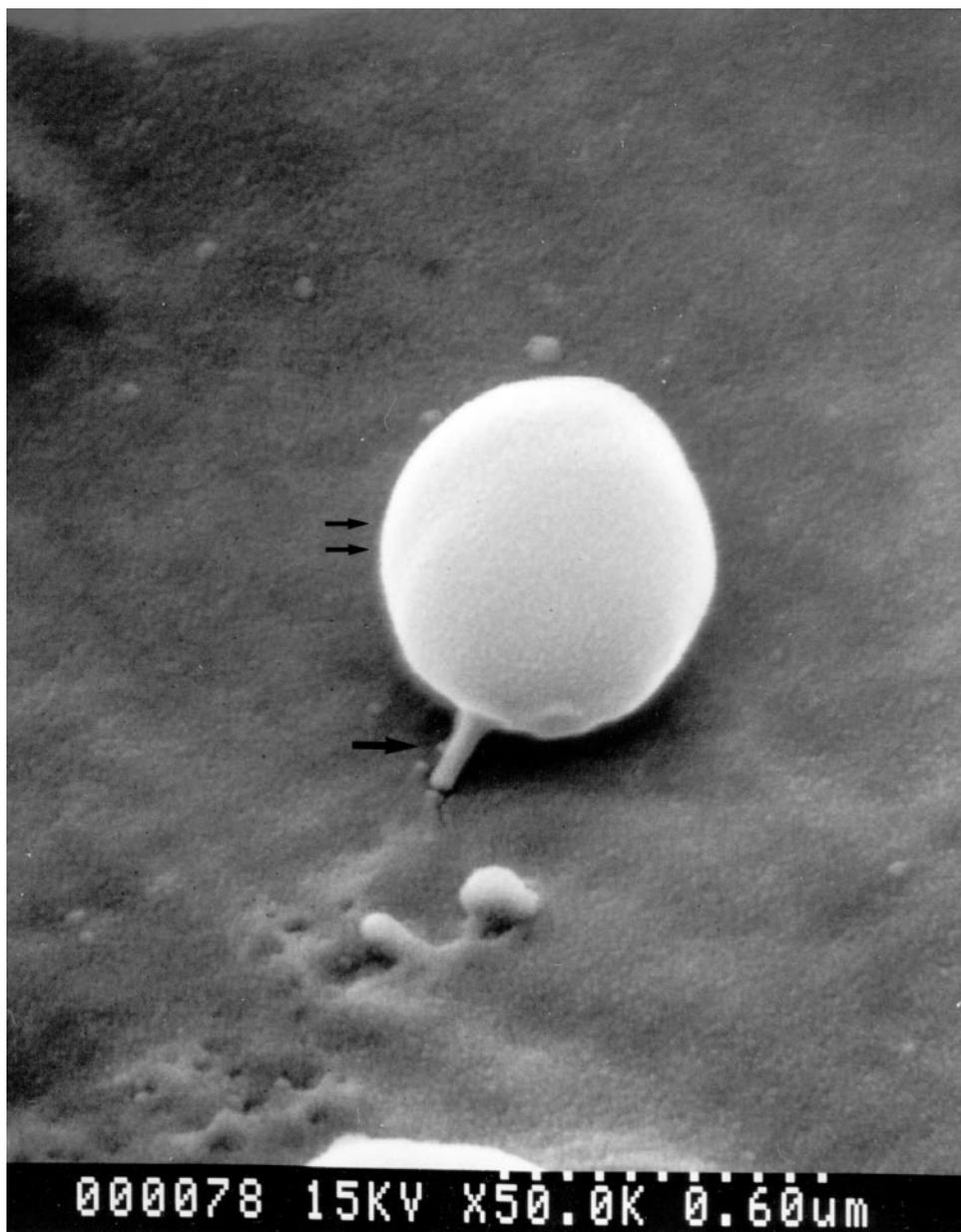
FIGURE 2. Leglike appendage (*single arrow*) of an *S. epidermidis* (*double arrow*) on an IOL, observed after 5 minutes of incubation. It seemed to measure approximately 0.16 μm in size. Original magnification, ×50,000.

000078 15KV X50.0K 0.60um
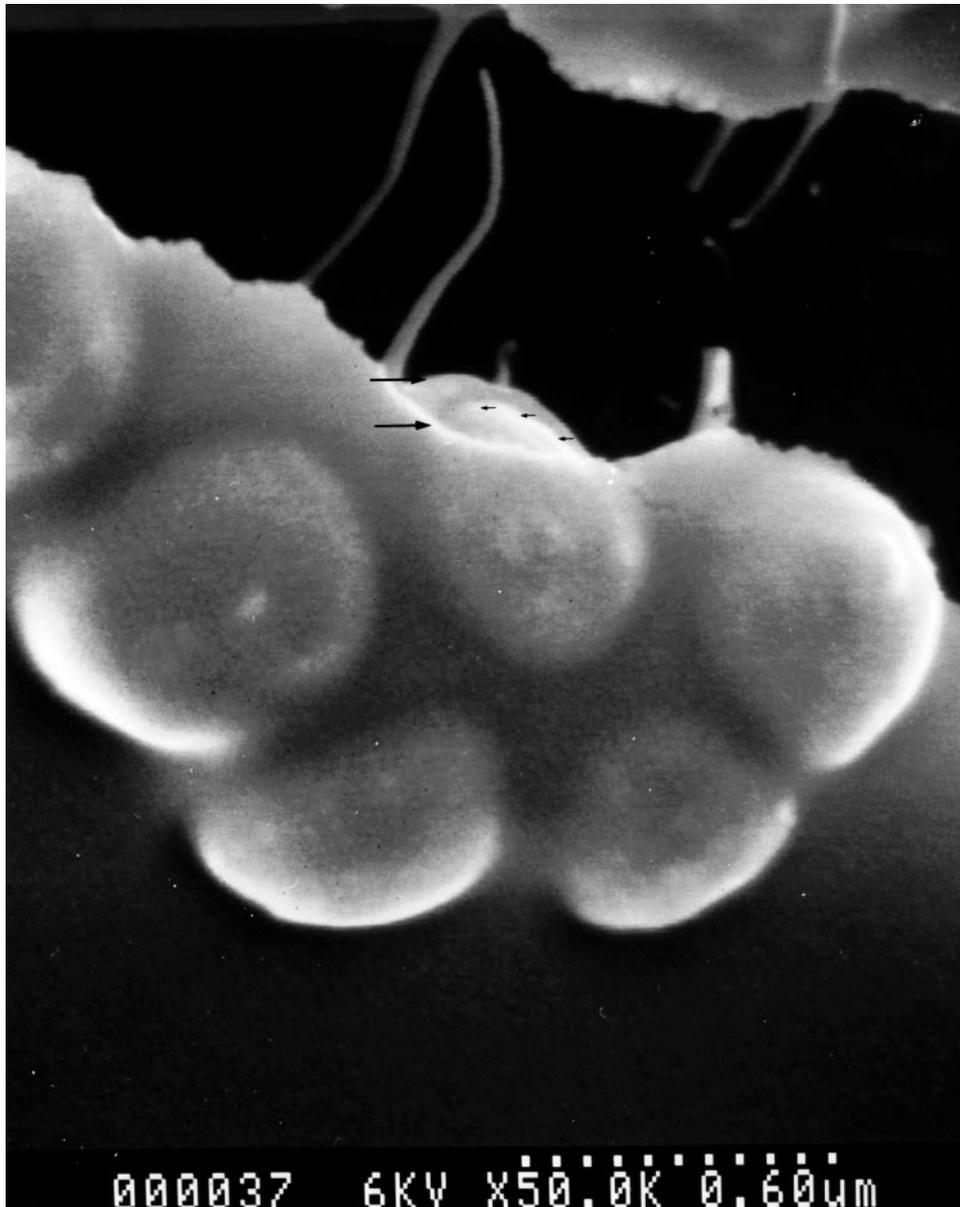
FIGURE 3. The adhesive layer was disrupted during sample examination, showing that bacterial colonies were literally coated on all sides with the layer. *Large arrows*: anterior and posterior borders of the slime layer; *small arrows*: the edge of the bacterium, which was completely embedded in the slime layer. Incubation time in the bacterial suspension: 1 hour. Original magnification, ×50,000.

of *ica* of approximately 685 to 687 bp, common to *icaA* and *icaB* from both *S. epidermidis* and *Staphylococcus aureus* (GenBank accession numbers AY138959 and AF086783, respectively; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).[18] Amplification mixtures were denatured at 95°C for 5 minutes, then subjected to 30 PCR cycles (denaturation: 94°C, 1 minute; annealing 55°C, 1 minute; extension 72°C, 1 minute). Positive controls included amplification of the *gyrA* or 16S-23S intergenic rRNA region.[18] PCR products were analyzed by electrophoresis on 0.8% agarose gels (Sigma-Aldrich, Lyon, France), purified with a kit (High-Pure; Roche Diagnostics, Mannheim, Germany) and sequenced using the listed PCR primers (Genome Express, Grenoble, France).

Scanning Electron Microscopy

Bacterial adherence was then investigated by SEM. The IOLs were first fixed with 2% (wt/vol) glutaraldehyde in filter-sterilized, 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature for 2 hours and then rinsed three times after 15 minutes in 0.15 M sodium cacodylate buffer. Next, a postfixation step was performed for 1 hour with 1% (wt/vol) osmium tetroxide in a 0.1 M sodium cacodylate buffer, followed by a quick rinse in distilled water. Fixed lenses were dehydrated step by step in ethanol. Samples were soaked, first in ethanol-water mixtures, with increasing ethanol concentrations (30%, 50%, 70%, 80%, and 95% of volume, successively), for 7 minutes each, then in pure ethanol for 5, 10, and 15 minutes, successively. They were then put in an ethanol bath that was allowed to evaporate. Dried samples were stuck on metal holders with double-sided scotch tape and finally coated in an evaporator with gold and palladium. Observations were usually performed at 5 kV with a scanning electron microscope (model S800; Hitachi, Tokyo, Japan; performed at the Scanning Electron Microscopy Centre for Applied Biology, Campus de la Doua, Lyon, France). Five samples were evaluated for each of the two groups of incubation periods (5 minutes or 1 hour). The entire surface of the IOL was examined.

RESULTS

Distribution of *ica*

The *S. epidermidis* strain N890074 was examined for the presence of *ica* by PCR amplification, using primers chosen to hybridize with *icaA* and *icaB*. N890074 was positive for *ica* (Fig. 1). The amplicon was sequenced, and the sequence was 100% identical with the published sequences in GenBank.
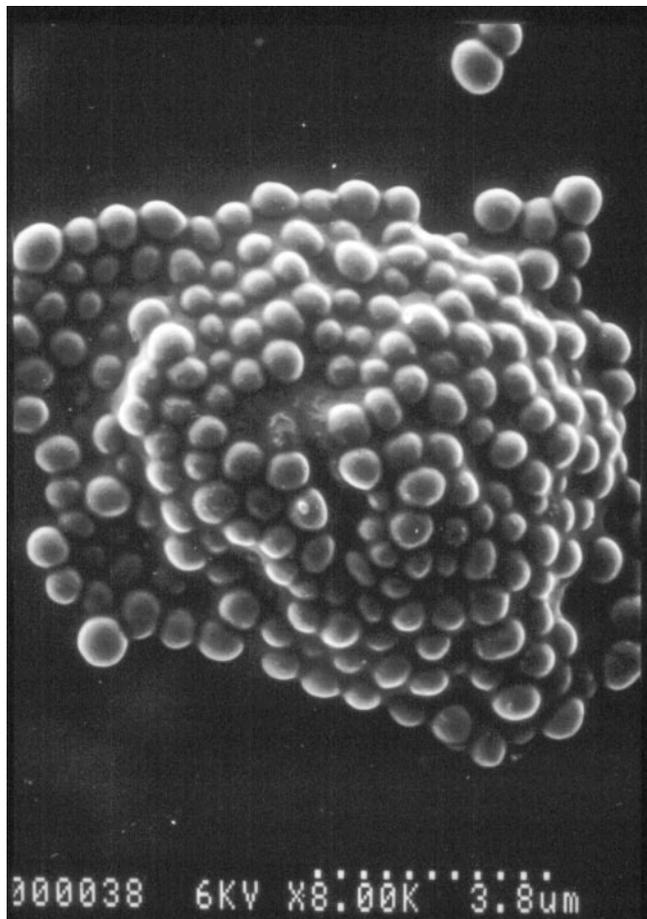
FIGURE 4. Bacteria appeared to accumulate on the IOL surface as microcolonies (clusters of bacteria) and a mucoid layer seemed to shroud bacteria. Incubation time in the bacterial suspension: 1 hour. Original magnification, ×8000.

Scanning Electron Microscopy

Our study was also designed to assess the morphologic features of coagulase-negative staphylococci that adhere to IOLs. Leglike appendages (Fig. 2) arose at the very beginning of the incubation. They were seen only in the samples with a 5-minute incubation period. According to several photographs, they measured apparently approximately 0.16 μm in length, although this could depend on the angle of the shot. These rod-shaped appendages appeared to project from the bacterial cell wall toward the IOL surface.

After long-lasting incubation (1 hour), bacteria were encased in an adhesive and mucoid material on the IOL surface. When this mucoid layer was disrupted by the electron's impact during the course of sample examination (Fig. 3), bacterial clusters appeared clearly to be coated on all sides with the gelatinous material. That is most likely why bacteria were found to accumulate on the lens surface as microcolonies and why a layer of adhesive material was visible all around the cells (Fig. 4). Observations were usually made above and at right angles to the sample surface. In the assay shown in Figure 5, the IOL was tilted. From this favorable angle, only the outer halves of the *S. epidermidis* cells were exposed and visible at the sample surface, because a cloudy, mucoid layer covered the remaining of the bacteria next to the IOL. The bacteria were literally embedded, and slime was the likely adhesive material. This photograph shows as well that all the slime was not yet present after an incubation of 1 hour, because only the outer halves of *S. epidermidis* cells were covered. Bacterial colonization of the polymer had just begun.

Moreover, the leglike appendages and the adhesive layer seemed to develop successively. Although the former were conspicuous after incubation times as short as 5 minutes (Fig. 2), bacteria probably became coated with slime at a subsequent stage, because photographs showed a gelatinous layer only after 1-hour incubations (Figs. 3, 4, 5). Indeed, Figure 2 does not show a layer of slime around the bacteria, in contrast to Figures 3, 4, and 5. Besides, leglike appendages were no longer detected after long-lasting incubations, because they were probably hidden within the depth of the slime layer.

DISCUSSION

Coagulase-negative staphylococci are currently recognized as important etiological agents of endophthalmitis, a nosocomial infection that follows the implantation of IOLs.[19] It seems likely that these microorganisms opportunistically infect implanted medical devices, because they readily adhere to their surfaces. Indeed, Vafidis et al.[20] rubbed IOLs against conjunctiva for 5 seconds before incubating them in nutrient broth. In 26% of cases, *S. epidermidis* was found to be growing in the medium. The mechanisms by which bacteria adhere so quickly to the IOL surface therefore merit strong attention.

Bacterial adherence relates to the capacity of some microorganisms to bind in a relatively stable way onto a surface of varied nature. This property represents the first step in colonizing a territory. It involves extremely complex interactions that are unlikely to fall within the scope of any generally valid description. Bacterial adherence to IOLs varies significantly, depending on IOL surface characteristics,[2] but also depending on the strain of bacteria.[21] The bacterial surface may contain hydrophobic and hydrophilic sites, the latter including both positively and negatively charged groups.[22,23]

Biofilm formation is thought to be a two-step process that requires the adhesion of bacteria to a solid substrate followed by cell–cell adhesion, forming the multiple layers of the biofilm called slime.[12,14–16,24] In this in vitro study, SEM showed parts of the first and second step of biofilm formation for the tested strain, containing the specific *ica* locus that encodes production of the bacterial antigens (both PS/A and PIA) mediating adherence to biomaterials and between the bacterial cells.[14,15]

The initial binding event is thus governed by the balance between three fundamental nonspecific physicochemical forces (van der Waals [hydrophobic] electrostatic and acid-base interactions), by the capsular polysaccharide/adhesin (PS/A), by one of several surface proteins (staphylococcal surface protein, fibrinogen binding protein, matrix protein binding), or by a combination of these.[16,20] This first phase takes place immediately,[24] which is comparable to our results. Although slime secretion is quite well documented, the production of leglike appendages is by far more intriguing. It is not clear whether they represent cell wall material projected quickly toward the IOL surface by the bacteria to strengthen their initial binding or an early image of slime secretion. The time delay remains an important, although as yet unknown, factor. Specific factors are involved in primary attachment of *S. epidermidis*. Some bacterial proteins are precisely described to be organized in a fimbriae-like structure.[16] Moreover, the first step of adhesion can induce modifications of genomic expression, as has been shown for *S. epidermidis*.[14] Bacteria could thus acquire new adhesive structures—pili, lateral flagella, exopolymers[25]—that could resemble leglike appendages. Recently, Vidal et al.[26] showed curli (a type of pili) overexpression by an *Escherichia coli* mutant strain, induced by the surface contact and regulated by microenvironmental sensors. Furthermore, Leatherwood[27] and Akin[28] and Akin and Amos[29] found rodlike appendages attaching rumen bacteria to cellulose or plant cell walls. Samples were fixed in glutaraldehyde and osmium tet-
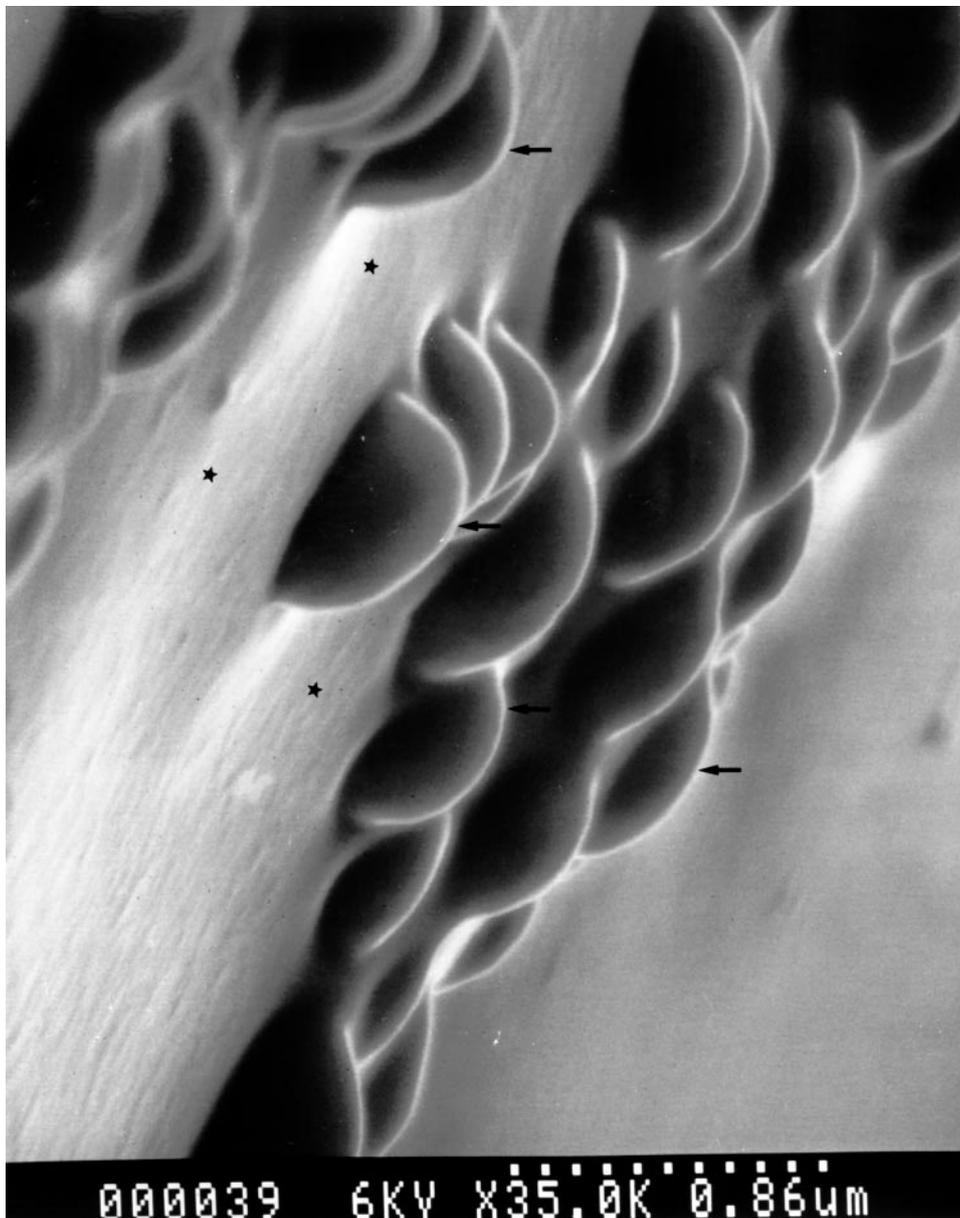
FIGURE 5. The IOL was tilted before taking the photograph. Only the outer halves of *S. epidermidis* cells (*black arrows*) were visible at the sample surface, because a mucoid layer covered the remaining part of bacteria next to the IOL (★). This material was probably slime. Incubation time in the bacterial suspension: 1 hour. Original magnification, ×35,000.

roxide, as in our own study. Moreover, according to some photomicrographs,[29] a single rumen bacterium could be anchored by as many as four or five stalks, each of them being approximately 0.16 μm long, as are the leglike appendages of the bacteria in our study.

The second, more prolonged, phase of the adherence process is mediated by the bacterial production of a polysaccharide glycocalyx (referred to as slime) on the IOL surface.[5,17] Photographs proved the existence of slime, secreted by the *S. epidermidis* strain N890074. Some bacterial strains produce an extensive amorphous extracellular polysaccharide material called slime,[30] which can vary from a few micrometers to a few millimeters in thickness.[31] It usually takes a few hours for the biofilm to form completely,[24] as is confirmed by the present study (Fig. 5). The biofilm is composed of multilayered cell clusters embedded in slime.[16] PIA displays intercellular adhesion and is responsible for bacterial accumulation in cellular aggregates and biofilm formation.[14-16] In a population of 179 *S. epidermidis* strains, there was a significant positive association between biofilm-production and PIA expression; 86.8% of biofilm-producing *S. epidermidis* strains produced PIA. This was detected with a specific coagglutination assay.[32] In con-

trast, in the same study, 88.6% of the biofilm-negative isolates did not express PIA. PIA appears essential for biofilm accumulation in most clinical *S. epidermidis* isolates. Moreover, PIA has a major role in the ability of *S. epidermidis* strains to hemagglutinate,[33] and a linear relation has been found between hemagglutination and biofilm production.[16,34]

The *ica* locus of *S. epidermidis* has been shown to encode production of both PS/A and PIA and has been associated with initial bacterial adherence, biofilm formation, and intercellular adhesion.[14,15] McKenney et al.[14] found that *ica*-positive strains of *S. epidermidis* adhered 3- to 10-fold more to silastic catheters during a 30-minute assay than did *ica*-negative strains. These results are consistent with those reported by Pinna et al.[24] The *ica* locus may represent an important genetic marker of *S. epidermidis* by promoting adherence to IOL surfaces and so, postoperative endophthalmitis.

The ability to produce slime is considered to be one of the virulence factors of staphylococci.[30,35] It is assumed that the bacteria producing the slime adhere better to smooth surfaces, above all to the surfaces of prosthetic devices implanted into organisms,[36] and that they become more resistant to antibiotics and to host responses.[7-13] These data suggest that slime-

mediated adherence may be a critical factor in the pathogenesis of *S. epidermidis* strains implicated in medical devices colonization. It would thus appear both as a colonization factor and a virulence factor. Investigations seeking to specify its role are worth considering and justify further studies.

The SEM observations enabled the slime to be visualized concretely on IOLs. They showed that *S. epidermidis* cells could be anchored to IOL surfaces by various means, especially by leglike appendages (shown for the first time in ophthalmology) and by becoming embedded in or shrouded by a slime layer.

Acknowledgments

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