

Detection of *Staphylococcus aureus* and *Staphylococcus epidermidis* in Clinical Samples by 16S rRNA-Directed In Situ Hybridization

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Received 17 November 1998/Returned for modification 1 February 1999/Accepted 29 March 1999

Staphylococcus epidermidis and *Staphylococcus aureus* are the most common causes of medical device-associated infections, including septicemic loosening of orthopedic implants. Frequently, the microbiological diagnosis of these infections remains ambiguous, since at least some staphylococci have the capacity to reduce their growth rate considerably. These strains exhibit a small-colony phenotype, and often they are not detectable by conventional microbiological techniques. Moreover, clinical isolates of *S. aureus* and *S. epidermidis* adhere to polymer and metal surfaces by the generation of thick, multilayered biofilms consisting of bacteria and extracellular polysaccharides. This study reports improved detection and identification of *S. aureus* and *S. epidermidis* by an in situ hybridization method with fluorescence-labeled oligonucleotide probes specific for staphylococcal 16S rRNA. The technique has proven to be suitable for the in situ detection of staphylococci, which is illustrated by the identification of *S. epidermidis* in a connective tissue sample obtained from a patient with septicemic loosening of a hip arthroplasty. We also show that this technique allows the detection of intracellularly persisting bacteria, including small-colony variants of *S. aureus*, and the differentiation of *S. epidermidis* from other clinically relevant staphylococci even when they are embedded in biofilms. These results suggest that the 16S rRNA in situ hybridization technique could represent a powerful diagnostic tool for the detection and differentiation of many other fastidious microorganisms.

Staphylococci are the most important pathogens in polymer-associated infections. They cause a wide range of polymer-related infections as well as infections associated with prosthetic joints and other implanted biomaterials (13, 21, 30). Although staphylococci are easy to identify by classical microbiological techniques, in some cases the correct species diagnosis is difficult. This might be due to the fact that at least some clinical staphylococcal isolates can differ in a wide range of phenotypic properties, leading to misinterpretations of biochemical tests (10, 11, 23, 29). In *Staphylococcus aureus*, the appearance of small-colony variants (SCVs) has been associated with recurrent and persistent infections (29). SCVs are characterized by a reduced growth rate, diminished exoprotein production, and increased resistance to aminoglycosides. Moreover, these bacteria were found to persist intracellularly. It was shown that this phenotype is due to defects in the hemin and menadione metabolism (5, 29, 33). The microbiological identification of SCVs is often complicated by the reduced growth rate and altered cell wall and exoprotein production. In *S. epidermidis*, variation of phenotypic features, including variation of enzyme expression, has also been described (8–11, 27, 35).

In the pathogenesis of foreign body-related infections caused by *S. epidermidis*, adhesion of the bacteria to the biomaterials is an essential step (12, 28). Scanning electron microscope investigations of polymer devices revealed that multilayered cell clusters of staphylococci are embedded in a thick

matrix of slime substance (14, 28). This process is suggested to occur in two stages: (i) a rapid initial attachment of the bacteria to the polymer surfaces is followed by (ii) a cell proliferation process and the production of an extracellular polysaccharide substance which mediates the intercellular adherence of the bacteria and the accumulation of a multilayered biofilm (17–19, 24, 25, 31). The production of this substance, termed the polysaccharide intercellular adhesin, was found to be associated with the presence and expression of the *ica* operon, which is widespread in clinical *S. epidermidis* isolates (19, 35).

This study was aimed at the development of an improved identification method for clinically important staphylococcal species by using a 16S rRNA-based oligonucleotide in situ hybridization technique which also detects biofilm-forming staphylococci and small-colony phenotypes in tissue samples. The study demonstrates the potential of the technique for the detection of other fastidious microorganisms which often remain unidentified by classical microbiological approaches.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1.

16S rRNA-targeted DNA probes. DNA oligonucleotide probes were synthesized and labeled with Cy3 or fluorescein by MWG Biotech GmbH, Ebersberg, Germany. The probe SEP-1, which is specific for *S. epidermidis*, *S. saccharolyticus*, *S. caprae*, and *S. capitis*, was described by Zakrzewska-Czerwinska et al. (34). The DNA probe EUB338, which is specific for all eubacteria, was reported by Amann et al. (2). The *S. aureus*-specific oligonucleotide probe SA-P1 was described by Bentley et al. (7). All oligonucleotide sequences are shown in Fig. 1.

RNA preparation for 16S rRNA hybridization. Overnight cultures were diluted 1:40 in 10 ml of brain heart infusion broth (Difco) and grown at 37°C in a shaking water bath to the mid-log phase. Bacterial cells were harvested by centrifugation for 5 min at 4,000 rpm. The pellet was resuspended in 500 µl of protoplast buffer containing 15 mM Tris-HCl (pH 7.5), 0.45 M sucrose, 8 mM EDTA (pH 8.0), and 100 µl of lysostaphin (500-µg/ml stock) (Sigma, Deisenhofen, Germany). The mixture was chilled for 20 minutes on ice and then

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TABLE 1. Bacterial strains

Strain ^a	Source ^b or reference
1. <i>S. epidermidis</i> RP62A (ATCC 35984)	ATCC
2. <i>S. epidermidis</i> 163	Clinical isolate
3. <i>S. epidermidis</i> 165	Clinical isolate
4. <i>S. epidermidis</i> 161	Clinical isolate
5. <i>S. epidermidis</i> 183	Clinical isolate
6. <i>S. epidermidis</i> 195	Clinical isolate
7. <i>S. epidermidis</i> 9	Clinical isolate
8. <i>S. warneri</i> DSM 20316	DSM
9. <i>S. warneri</i> 193	Clinical isolate
10. <i>S. aureus</i> 8325/4 (NCTC 8325)	NCTC
11. <i>S. aureus</i> 173	Clinical isolate
12. <i>S. aureus</i> 175	Clinical isolate
13. <i>S. aureus</i> 3	Clinical isolate
14. <i>S. saprophyticus</i> DSM 20229	DSM
14. <i>S. haemolyticus</i> DSM 20263	DSM
15. <i>S. cohnii</i> 194	Clinical isolate
16. <i>S. cohnii</i> DSM 20260	DSM
17. <i>S. carnosus</i> TM300	28
18. <i>S. lugdunensis</i> DSM 4804	DSM
19. <i>Enterococcus faecalis</i> DSM 2570	DSM
20. <i>E. coli</i> K-12 strain C600	
22. <i>Klebsiella pneumoniae</i> IA565	Clinical isolate
23. <i>Proteus mirabilis</i> 102	Clinical isolate
24. <i>S. typhimurium</i> C17	Clinical isolate
25. <i>Citrobacter freundii</i> 3009	Clinical isolate
26. <i>S. capitis</i> DSM 20326	DSM
27. <i>S. aureus</i> 453	Clinical isolate
28. <i>C. albicans</i> ATCC 44808	ATCC
29. <i>S. aureus hemB</i> mutant	28
30. <i>E. coli</i> K-12 strain HB101	

^a Numbers correspond to those in Fig. 3a.

^b ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; NCTC, National Collection of Type Cultures.

incubated for 5 to 20 min at 37°C. Following the addition of 500 µl of lysis buffer (30 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM EDTA [pH 8.0], 1% sodium dodecyl sulfate [SDS], 100 µg of proteinase K per ml), the suspension was twice frozen at -80°C and thawed at room temperature. Following the last thawing, the suspension was incubated for 1 h at 37°C, and phenolchloroform-isoamyl alcohol extraction was performed five times. The RNA was precipitated with 5 M sodium chloride and 2 volumes of chilled 96% ethanol. Following centrifugation, the RNA pellet was rinsed with 80% ethanol and air dried.

DIG labeling of oligonucleotides for dot blot hybridization. For digoxigenin (DIG) labeling, the DIG Oligonucleotide 3'-End Labeling Kit (no. 1362372; Boehringer, Mannheim, Germany) was used. The labeling procedure was performed according to the instructions of the manufacturer.

Dot blot hybridization with DIG-labeled oligonucleotides. Ten micrograms of RNA was blotted onto a nylon membrane (PALL Biodyne B membrane) by using a dot blot apparatus (Schleicher & Schuell SRC 96 D Minifold I Dot Blotter) and UV fixed. For the hybridization procedure high-SDS buffer containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 7% SDS, 50 mM sodium phosphate (pH 7.0), 2% blocking reagent (Boehringer), and 0.1% *N*-lauroylsarcosine was used. All buffers and solutions were used according to the instructions of the supplier. The hybridization temperature was 42°C. Washing of the membrane was performed by using a high-stringency washing buffer containing 0.1% SDS and 0.1× SSC at 50°C. Detection of the DIG-labeled oligonucleotide was performed by using alkaline phosphatase-labeled anti-DIG antibody Fab fragments (Boehringer) and the chemiluminescent substrate CSPD (Boehringer) by the procedure recommended by the supplier. The membrane was exposed to Hyperfilm-ECL (Amersham) for 1 to 60 min.

Bacterial cell fixation and permeabilization for whole-cell hybridization. Lysis of the bacterial cells was performed as described previously by Matsuhisa et al. (26) with some modifications. Briefly, bacteria were grown to exponential phase and harvested by centrifugation. The pellet was resuspended and washed in phosphate-buffered saline (PBS) (130 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.4). Cells were fixed in 4% paraformaldehyde-PBS for 1 h, fixed again in 70% ethanol for 30 min, and stored in a 1:1 mixture of ethanol and PBS as described previously (2). For the hybridization, 1 µl of bacterial cells was dropped onto a microscope slide and air dried. For permeabilization, the cells were treated with an enzyme mix (750 µg of lysostaphin per ml, 5 mg of lysozyme per ml, 50 mM sodium phosphate, 0.05% saponin) for 1 h at 37°C.

A

Strain	Sequence	Accession number
<i>S. epidermidis</i>	TCCT CTGACCCCTCTAGAGATAGAGT	ITT L37605
<i>S. saccharolyticus</i>	TCCT CTGACCCCTCTAGAGATAGAGT	ITT L37602
<i>S. capitis</i>	TCCT CTGATCCCTCTAGAGATAGAGT	ITT L37599
<i>S. auricularis</i>	TCCT TTGACCGCTCTAGAGATAGAGT	ITT L37598
<i>S. warneri</i>	TCCT TTGACCGCTCTAGAGATAGAGT	ITT L37603
<i>S. haemolyticus</i>	TCCT TTGACAACTCTAGAGATAGAGC	ITT L37600
<i>S. aureus</i>	TCCT TTGACAACTCTAGAGATAGAGC	ITT L37597
<i>S. hominis</i>	TCCT TTGACCCCTCTAGAGATAGAAAG	ITT L37601
<i>S. saprophyticus</i>	TCCT TTGAAAACCTCTAGAGATAGAGC	ITT L37596

B

Strain	Sequence	Accession number
<i>S. aureus</i>	CGAA CGGACGAGAAGCTTG CTCTCTCT	GAT L37597
<i>S. epidermidis</i>	CGAA CAGACGAGGAGCTTGCTCCTCT	GAC L37605
<i>S. capitis</i>	CGAA CAGACGAGGAGCTTGCTCCTCT	GAC L37599
<i>S. warneri</i>	CGAA CAGATAAGGAGCTTGCTCCTTT	GAC L37603
<i>S. haemolyticus</i>	CGAA CAGACAAGGAGCTTGCTCCTTT	GAC L37600
<i>S. hominis</i>	CGAA CAGACGAGGAGCTTGCTCCTTT	GAC L37601
<i>S. saprophyticus</i>	CGAA CAGATAAGGAGCTTGCTCCTTT	GAC L37596

FIG. 1. (A) Sequence alignment of the region in the 16S rRNA genes of staphylococci corresponding to nucleotides 1014 to 1043 of the *E. coli* 16S rRNA. The target region of the oligonucleotide probe SEP-1 is boxed. Nucleotides different from those in the *S. epidermidis* sequence are in boldface. (B) Sequence alignment of the region in the 16S rRNA genes of staphylococci corresponding to nucleotides 66 to 95 of the *E. coli* 16S rRNA. The target region of the oligonucleotide SA-P1 is boxed. Nucleotides different from those in the *S. aureus* sequence are in boldface.

Whole-cell hybridization. Fifteen microliters of hybridization solution (0.9 M NaCl, 20 mM Tris-HCl [pH 7.5], 0.001% SDS, 43% formamide, 50 ng of Cy3- or fluorescein-labeled probe) was applied to microscope slides and incubated for 3 h at 43°C in an isotonicly equilibrated humid chamber (6). Removal of the unbound probe and washing were performed at 43°C as described by Amann et al. (3). The slides were rinsed briefly with distilled water, air dried, and mounted with Citifluor solution (Citifluor Ltd., London, United Kingdom). Fluorescence was detected by using an Axioplan microscope (Zeiss, Oberkochen, Germany) equipped with an epifluorescence unit.

Detection of *S. epidermidis* in biofilms. The biofilm-producing strains *S. epidermidis* RP62A and *S. aureus* 453 were used. Bacterial strains were grown overnight in chamber slides (Lab-Tek II chamberslide no. 154534; Nalge Nunc International, Naperville, Ill.) in Trypticase soy broth at 37°C. After decanting, the supernatant slides were washed three times with PBS to remove all nonadhering bacteria. Bacterial biofilms were fixed by air drying and treated for hybridization as described above for the whole-cell hybridization assay.

Detection of an *S. aureus hemB* mutant in a human endothelial cell line by in situ hybridization. For the intracellular persistence assay, the following bacterial strains were used: *S. aureus* 8325/4 (noninvasive), an invasive *S. aureus* 8325/4 *hemB* mutant (33), *Salmonella typhimurium* C17 (invasive positive control) (15), and *Escherichia coli* HB101 (noninvasive negative control).

The human endothelial cell line E.Ahy 926 was grown to a confluent monolayer in RPMI medium with 10% fetal calf serum and 2 mM glutamine in chamber slides (Lab-Tek II chamberslide no. 154534). Washed bacteria were adjusted to nearly equal numbers for each strain and added to the washed monolayers. The infected monolayers were incubated for 3.5 h (2.5 h for the *hemB* mutant) at 37°C in 5% CO₂ to allow adhesion and invasion of the bacteria. The monolayers were washed three times with PBS. In order to remove all extracellular bacteria, 1 ml of medium containing 10 µg of lysostaphin (Sigma) per ml was added in case of the *S. aureus* strains. Extracellular *S. typhimurium* and *E. coli* were removed by adding 100 µg of gentamicin/ml. Incubation in both cases was for 30 min at 37°C. The monolayers were washed three times with PBS and fixed for 20 min in 4% freshly prepared paraformaldehyde at room temperature. Dehydration was performed stepwise for 5 min in 50, 70, and 100% ethanol. For the detection by the fluorescent oligonucleotide probes, the fixed and dehydrated monolayers were treated as described above for the permeabilization for whole-cell hybridization. The conditions for the in situ hybridization were exactly the same as for the whole-cell hybridization assay.

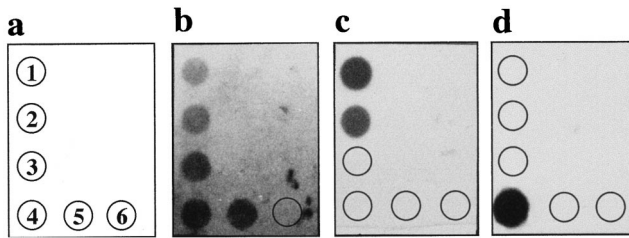


FIG. 2. RNA dot blot hybridization with *S. epidermidis* 163 (1), *S. capitis* DSM 20326 (2), *S. warneri* DSM 20316 (3), *S. aureus* 8325/4 (4), *S. saprophyticus* DSM 20229 (5), and *C. albicans* ATCC 44808 (6). The oligonucleotide probes were labeled with DIG, and hybridizations were performed as described in Materials and Methods at 42°C in a buffer containing 50% formamide. (a) Strain numbers; (b) hybridization with probe EUB338; (c) hybridization with probe SEP-1; (d) hybridization with probe SA-P1.

In situ hybridization of paraffin-embedded tissue samples. To remove paraffin, the tissue sections were treated twice with Rotihistol (Roth, Karlsruhe, Germany) for 10 min and then with Rotihistol-ethanol (1:1) for 10 min. For the detection by the fluorescent oligonucleotide probes, the tissue sections were treated as described above for the permeabilization for whole-cell hybridization. The conditions for the in situ hybridization were exactly the same as for the whole-cell hybridization assay.

RESULTS AND DISCUSSION

Specificities of the oligonucleotide probes. The detection and identification of microorganisms based on their 16S rRNAs have many advantages. First, each bacterial cell contains multiple copies of the 16S rRNA in its ribosomes. Hence, the technique is sensitive enough to detect single bacterial cells. Second, 16S rRNA genes are highly conserved throughout bacterial evolution. They consist of regions which are common to all eubacteria and other regions which are extremely species specific. By using the appropriate gene probes, it is possible either to detect any bacterial pathogen or, when highly specific probes are used, to identify single bacterial species. Also, this technique allows for the identification of microorganisms independently of bacterial growth rates and metabolic activities. This is a special advantage for the detection of dormant and metabolically inactive bacteria, since the number of ribosomes is not significantly affected in such organisms. This article reports the development of improved diagnostic tools for both *S. aureus* and *S. epidermidis*, which are the most common causative agents of infections in orthopedic implants (30).

For the detection of *S. aureus* and *S. epidermidis*, two oligonucleotide probes which are directed against specific regions in the 16S rRNA were used. The nucleotide sequence of the *S. epidermidis*-specific probe SEP-1 was described by Zakrzewska-Czerwinska et al. (34). For the *S. aureus*-specific probe SA-P1, the sequence published by Bentley et al. (7) was used. Figure 1 shows an alignment of the 16S rRNA genes of different *Staphylococcus* species and the positions of the SEP-1 and SA-P1 probes.

In a first set of experiments, the specificities of the gene probes used in this study were analyzed by dot blot hybridizations (Fig. 2 and 3). To this end, RNAs of (i) different staphylococcal species, (ii) a range of nonrelated bacteria, and (iii) *Candida albicans* were isolated, dotted onto nylon membranes, and hybridized with the appropriate DIG-labeled gene probes. In a control experiment, the stability and the amount of the filter-immobilized RNA were determined by using the oligonucleotide probe EUB338 (2), which binds to the 16S rRNAs of all eubacteria (Fig. 3b) but not to the rRNA of *C. albicans* (Fig. 2b).

Figure 1A shows that the sequence of the SEP-1 probe

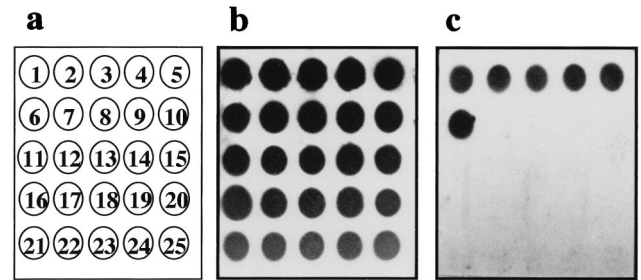


FIG. 3. RNA dot blot hybridizations with bacterial strains listed in Table 1. The oligonucleotide probes were labeled with DIG, and hybridizations were performed as described in Materials and Methods at 42°C in a buffer containing 50% formamide. (a) Strain numbers, corresponding to the first 25 strains, respectively, in Table 1; (b) hybridization with probe EUB338; (c) hybridization with probe SEP-1.

differs from those of most staphylococcal species by more than two nucleotides. In the cases of *S. capitis* and *S. warneri*, however, the SEP-1 probe contains only one and two nucleotide mismatches, respectively. As expected, the *S. epidermidis*-specific probe, SEP-1, hybridized to the RNAs from the *S. epidermidis* isolates but not to RNAs isolated from all of the other bacterial species analyzed in this experiment (Fig. 3c). The data also show that it was not possible to distinguish between *S. epidermidis* and the very closely related species *S. capitis* (Fig. 2c). Since the *S. epidermidis*-specific gene probe contains only one base exchange in comparison with the corresponding *S. capitis* 16S rRNA region, *S. capitis* strains were misidentified as *S. epidermidis*. This cross-hybridization of the SEP-1 gene probe with *S. capitis*, *S. saccharolyticus*, and *S. caprae* has also been described recently by Zakrzewska-Czerwinska et al. (34). *S. capitis*, *S. saccharolyticus*, and *S. caprae* have been shown to have a certain pathogenic potential (e.g., *S. caprae* can be associated with bone and joint infections in humans [20, 32]), but clearly, they are rarely isolated human pathogens (1, 22), whereas the vast majority of this type of infections are caused by *S. epidermidis*. Therefore, it is unlikely that the cross-reactivity of the SEP-1 probe will pose a real problem, particularly as the treatment strategy would be the same as that for an *S. epidermidis* infection. The *S. aureus*-specific oligonucleotide probe SA-P1 revealed positive signals only with the RNA isolated from *S. aureus* and not with that from any other species tested in this study (Fig. 2d). Thus, both oligonucleotide probes proved to be suitable for the identification of *S. aureus* and a group of four coagulase-negative staphylococci, including *S. epidermidis*, and for their discrimination from a range of other bacterial species. However, it should be noted that an extensive study involving a broad range of related and nonrelated bacterial species is required to completely exclude possible unspecific reactions of the oligonucleotide probes described here.

Fluorescent hybridization of whole bacterial cells. The goal of the experiments described next was to establish a 16S rRNA-based in situ hybridization method that would allow for an improved diagnosis of staphylococcal infections. First, it was important to show that staphylococcal cells can be sufficiently permeabilized to allow for intracellular hybridization with fluorescence-labeled oligonucleotide probes. Next, we attempted to answer the question of whether staphylococci can be reliably differentiated by this technique. To address these issues, different staphylococcal species were analyzed in a whole-cell hybridization assay. The microorganisms were grown in liquid cultures to the exponential phase, harvested, and treated as described in Materials and Methods. For the detection of *S.*

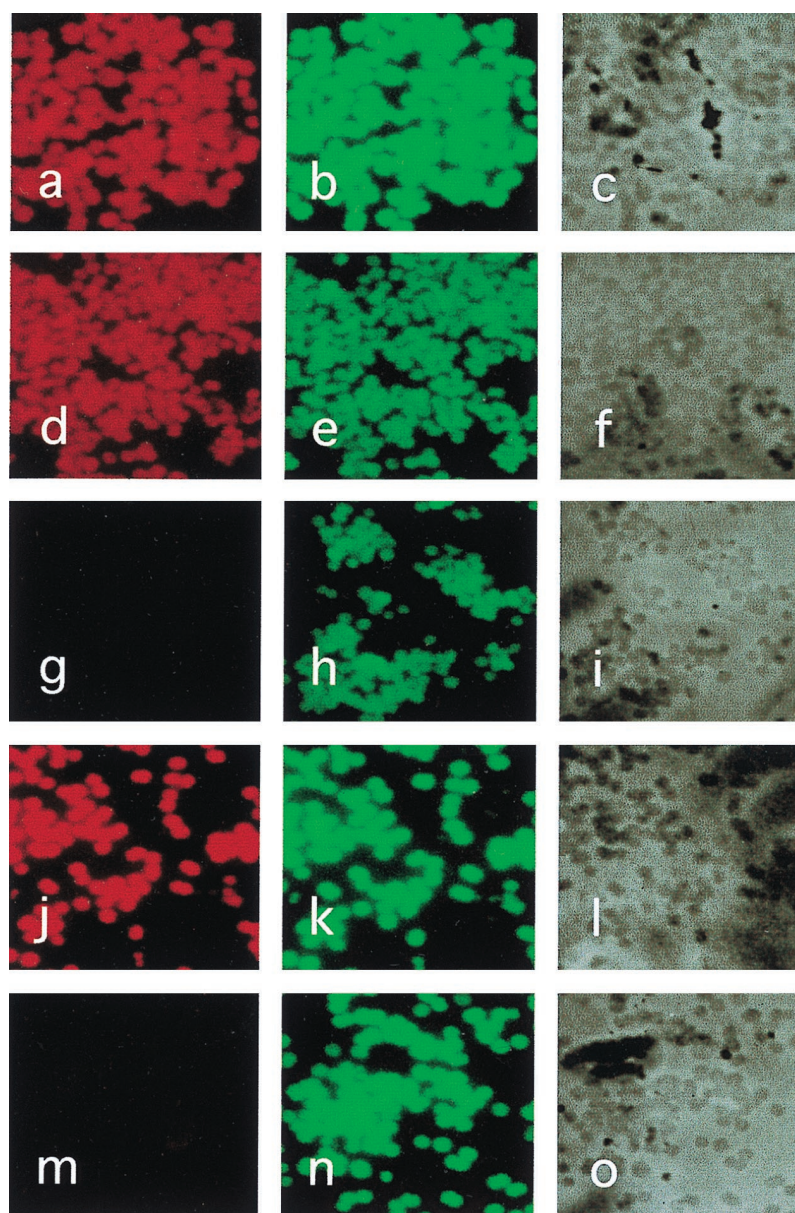


FIG. 4. Epifluorescence micrographs of *S. epidermidis* RP62A (a to c), *S. capitis* DSM 20326 (d to f), *S. warneri* DSM 20316 (g to i), *S. aureus* 8325/4 (j to l), and *S. epidermidis* RP62A (m to o) under high-stringency conditions (43°C, 43% formamide). The SEP-1 probe (a, d, and g), the SA-P1 probe (j and m), the EUB338 probe (b, e, h, k, and n), or phase contrast (c, f, i, l, and o) was used.

aureus and *S. epidermidis*, the Cy3-labeled oligonucleotide probes SA-P1 and SEP-1 were used. The FLUOS-labeled probe EUB338 was used as a positive control specific for all eubacterial species. The results obtained by fluorescence microscopy can be summarized as follows: (i) hybridization with the positive control (EUB338) revealed that all eubacterial cells are visualized by this technique (Fig. 4b, e, h, k, and n), (ii) the SEP-1 probe specifically stained all *S. epidermidis* and *S. capitis* cells (Fig. 4a and d), and (iii) with SEP-1, no specific fluorescence signals were obtained for *S. warneri* (Fig. 4g) and *S. aureus*, *S. haemolyticus*, and *S. saprophyticus* (data not shown). We concluded from these data that the two-nucleotide mismatch of the SEP-1 probe with the *S. warneri* target sequence appeared to be sufficient for a reliable discrimination between *S. epidermidis* and other staphylococcal species (with

the exception of *S. capitis*). Finally, the *S. aureus*-specific probe SA-P1 was found to specifically label *S. aureus* cells, whereas *S. epidermidis* and other staphylococci remained unstained (Fig. 4j and m).

Obviously, a gene probe capable of identifying all staphylococcal species at the genus level is highly desirable. In the absence of such a reagent, we are restricted to the identification of bacterial infections with the eubacterial gene probe EUB338 and the subsequent discrimination of *S. aureus* and *S. epidermidis* with specific gene probes. There is a clear need for the development of additional gene probes which could be used in combination for the identification of any bacterial pathogen.

Detection of *S. epidermidis* in bacterial biofilms. The adherence and formation of biofilms on smooth surfaces is an es-

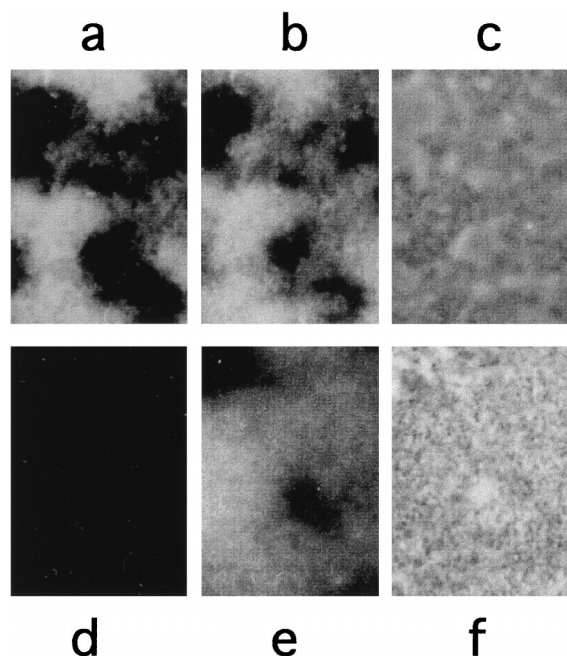


FIG. 5. Detection of staphylococci in biofilms. Phase-contrast (c and f) and epifluorescence (a to e) micrographs of *S. epidermidis* RP62A (a, b, and c) and *S. aureus* 8325/4 (d, e, and f) in biofilms following hybridization with the Cy3-labeled probe SEP-1 (a and d) and the FLUOS-labeled probe EUB338 (b and e) under high-stringency conditions (43°C, 43% formamide) are shown.

essential step in the pathogenesis of staphylococcal infections. Therefore, we decided to extend the approach described above to address the question of whether it is possible to identify staphylococci in biofilms. In these experiments, the biofilm-positive strain *S. epidermidis* RP62A, which produces the polysaccharide intercellular adhesin, was used. In parallel, an *S. aureus* clinical isolate which had been found to form biofilms on polystyrene surfaces was analyzed and served as a control. The fluorescent hybridization technique with the SEP-1 probe proved to be suitable for the detection of *S. epidermidis* cells, even when they were embedded in a thick matrix of extracellular polysaccharides (Fig. 5a). In the control experiment with the biofilm-forming *S. aureus* isolate, no specific staining was obtained. This finding confirms the specificity of SEP-1 for *S. epidermidis* and also rules out the possibility that the positive signal was generated by an unspecific binding to extracellular proteins or polysaccharides. We concluded from these data that SEP-1 is an appropriate diagnostic tool for the detection and differentiation of *S. epidermidis* isolates even when they form biofilms.

Detection of an *S. aureus* SCV phenotype by the fluorescent hybridization technique. The abilities of clinically relevant staphylococci both to generate biofilms and to vary their phenotypic properties (e.g., growth rate, antibiotic susceptibility, and adherence properties) have been suggested to contribute considerably to staphylococcal virulence. The occurrence of *S. aureus* SCVs has been associated with recurrent and persistent infections, especially in patients with osteomyelitis and in patients with orthopedic implants (29). SCVs are characterized by a reduced growth rate, an increased resistance to aminoglycosides, and intracellular persistence. In order to investigate whether SCVs can be detected by the direct hybridization technique, we did an invasion assay by using a site-directed *S. aureus hemB* mutant which exhibits all properties of a small-

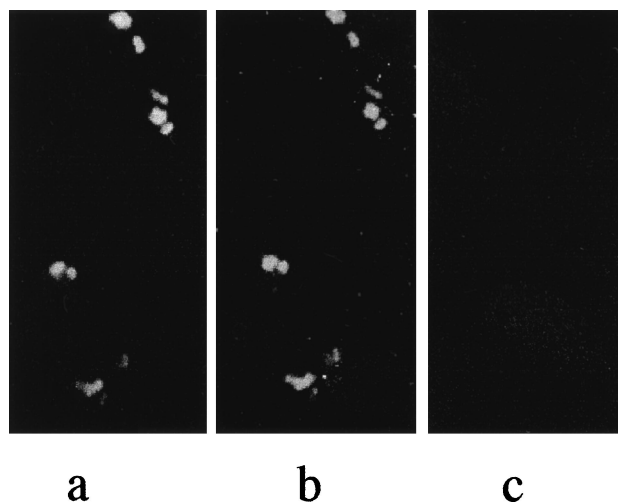


FIG. 6. Detection of intracellularly persisting bacterial cells of an *S. aureus hemB* mutant in the human endothelial cell line E.Ahy 926. (a) Hybridization with the FLUOS-labeled probe EUB338. (b) Hybridization with the Cy3-labeled probe SA-P1. (c) Negative control with the noninvasive strain *E. coli* HB101 probed with EUB338.

colony phenotype, including the invasion of endothelial cells (33). The human endothelial cell line E.Ahy 926 was incubated with the *S. aureus hemB* mutant as described in Materials and Methods. Thereafter, the fixed cells were hybridized with the oligonucleotide probes EUB338 and SA-P1. Fluorescence microscopy analysis revealed that the *S. aureus* SCVs can be detected by both the EUB338 and SA-P1 gene probes, even when the bacteria occur intracellularly (Fig. 6a and b). An *S. typhimurium* isolate which is invasive in epithelial cells was used as a positive control for the invasion assay (data not shown), and the noninvasive strain *E. coli* HB101 was used as a negative control (Fig. 6c). We conclude from this experiment that the 16S rRNA technique is indeed suitable for the detection of metabolically inactive bacteria even when they occur intracellularly. Therefore, the method gives the opportunity to improve the diagnosis of infections caused by staphylococcal SCVs.

In situ hybridization of a tissue section sample from a patient with hip arthroplasty loosening. A further important problem in the diagnosis of orthopedic staphylococcal infections is the evaluation of the microbiological results, especially when coagulase-negative staphylococci have been identified. Often it is difficult to decide whether an isolate represents the causative agent of an infection or an unspecific contamination from the skin or the environment. One possible way to overcome these problems is to use diagnostic tools which allow the direct detection and differentiation of the bacteria in situ (e.g., in tissues, smears, or biopsy material, etc.).

To conclude our study, we addressed the question of whether the 16S rRNA hybridization technique is also appropriate for the direct detection of staphylococci in tissues. We therefore used the method in a clinical case of hip arthroplasty loosening that was thought to be caused by *S. epidermidis*.

In a 65-year old female suffering from progressive loosening of an artificial hip arthroplasty, a replacement of the implant had to be performed. Since the clinical course was typical for an infection of the implant, specimens for microbiological investigations were obtained during surgery. Initially, microbiological cultivation failed to identify any causative agent. However, after prolonged cultivation (1 week) in Trypticase soy

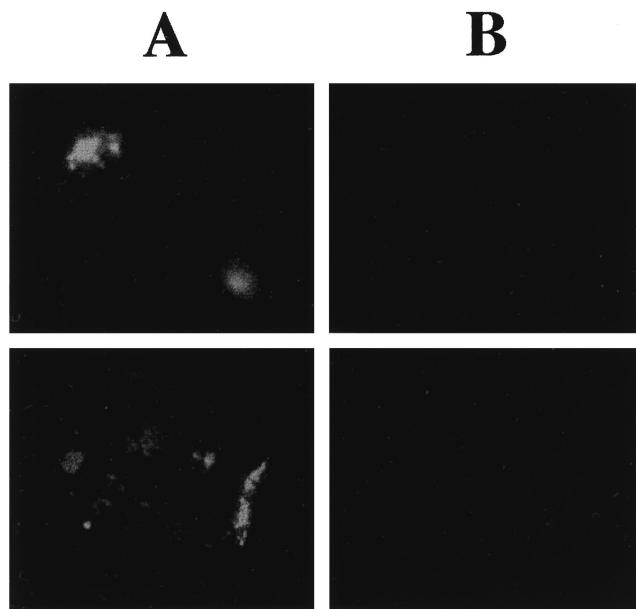


FIG. 7. In situ hybridization of connective tissue from a patient suffering from progressive loosening of an artificial hip arthroplasty with the *S. epidermidis* specific gene probe SEP-1 (A) and the *Legionella*-specific gene probe LEGPNE1 (16) as a negative control (B). Two different sectors of a sample that was obtained during replacement surgery are shown.

broth, *S. epidermidis* was identified in several specimens of the material. The *S. epidermidis* isolate was found to be resistant to oxacillin, gentamicin, and erythromycin. To decide whether the isolate represented the cause of the infection or a contamination, the 16S rRNA hybridization technique was used again. A tissue sample which had been obtained during the joint replacement surgery was fixed with paraformaldehyde and embedded in paraffin for thin-layer dissection. For the in situ hybridization with the fluorescence-labeled oligonucleotide probes, the tissue sections were treated as described in Materials and Methods. Both the EUB338 and SEP-1 probes were used in this experiment. In all tissue sections analyzed, several foci which gave signals with both oligonucleotide probes were identified. The foci were localized in the vicinity of blood vessels and resembled bacterial structures in size and shape. To ensure that the observed structures indeed represented bacteria, any unspecific reaction was excluded by probing another section with the *S. epidermidis*-specific SEP-1 probe and a *Legionella pneumophila*-specific probe, LEGPNE1 (16), as a negative control. No specific signal of the foci was obtained with the *Legionella*-specific probe in this control experiment (Fig. 7B). In contrast, the structures were strongly stained with the *S. epidermidis*-specific SEP-1 probe (Fig. 7A). In a prospective study using a mathematical model, it was shown recently that the investigation of at least three independent specimens gives results that are highly predictive of an infection (4). Although we had only one specimen at our disposal, we feel that the high number of the *S. epidermidis*-specific bacterial foci in the sample and their localization, combined with the microbiological cultivation results from several independent specimens, strongly suggest an involvement of an *S. epidermidis* infection in the loosening of the implant. The direct detection and identification of *S. epidermidis* in the infected tissue by this method support the clinical diagnosis that the loosening of the arthroplasty was caused by an infection.

In summary, this initial report shows that the in situ hybrid-

ization technique with 16S rRNA-directed oligonucleotide probes is suitable for the diagnosis of staphylococcal infections associated with orthopedic implants. It should be noted that, for several reasons, this method cannot be expected to replace conventional culture techniques. In those cases, however, where the standard cultivation fails to identify a causative infectious agent, the 16S rRNA in situ hybridization could represent a useful additional diagnostic tool. We have applied this method to the diagnosis of one clinical case of hip arthroplasty loosening. However, in order to explore its general usefulness in the diagnostic laboratory (in comparison with currently available culture techniques), a broad clinical study would be extremely desirable.

Encouraged by the results of this study, we believe that the method can be extended to allow for the identification of a wide range of other fastidious microorganisms (e.g., *Legionella*, *Burkholderia*, or streptococci). For this purpose, however, the method has to be optimized to make it practicable for use in the routine microbiological laboratory.

ACKNOWLEDGMENTS

We thank Rudolf I. Amann of the Max-Planck-Institut für Marine Biologie, Bremen, Germany, for helpful discussions and technical support with whole-cell hybridizations, and we thank Bernd Schmaußer of the Institut für Pathologie, Universität Würzburg, Würzburg, Germany, for carrying out the sectioning of paraffin-embedded tissue.

This work was supported by a grant from the BMBF (no. 01K19608), by the Graduiertenkolleg Infektiologie, and by the Fond der Chemischen Industrie.

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