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Comparison of Antibiotic Resistance of Blood Culture Strains and Saprophytic Isolates in the Presence of Biofilms, Formed by the Intercellular Adhesion (*ica*) Gene Cluster in *Staphylococcus epidermidis*

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Abstract To elucidate the question of whether biofilm formed by the intercellular adhesion (*ica*) gene cluster has influences on antibiotic resistance in *Staphylococcus epidermidis*, we compared 124 skin strains with strains isolated from 50 blood cultures that cause septicemic diseases. The results revealed that the blood culture isolates were more resistant to the antibiotics tested than the saprophytic isolates. Moreover, antibiotic multiresistance was more prevalent in the clinical isolates. In the blood culture isolates, 46% of the strains were resistant to three or more antibiotics, whereas only 12% of the saprophytic isolates were resistant to three or more antibiotics. Interestingly, these characteristics were highly correlated with the biofilm formed by the *ica* gene cluster. In biofilm-producing strains, 84% of the blood culture isolates and 44% of the saprophytic isolates were antibiotic multiresistant, whereas only 22% and 9%, respectively, were antibiotic multiresistant in biofilm-nonproducing strains. Additionally, in the biofilm-producing *ica*-positive strains, 89% of the blood culture isolates and 57% of the saprophytic isolates were antibiotic multiresistant. However, the rate of the antibiotic multiresistance in the *ica*-negative strains was very low, thus indicating that the biofim formed by the *ica* gene cluster in *S. epidermidis* is an important pathogenic factor in association with the antibiotic multiresistance.

Key words: Biofilm, intercellular adhesion (*ica*) gene cluster, *Staphylococcus epidermidis*, antibiotic multiresistance

Antibiotic therapy against an established biofilm often fails unless the infected implant is removed, despite the use of drugs that are highly active in standard *in vitro* susceptibility tests [16]. Previous studies have examined the antimicrobial susceptibility of *S. epidermidis* [6, 19] and *S. aureus* [6, 36] biofilms formed on orthopedic biomaterials.

S. epidermidis has received little attention so far for its role in infections. However, it has been recently observed that infections of implanted plastic devices by coagulase-negative staphylococci, in particular *S. epidermidis*, have become a major cause of septicemia in human patients [23]. The organism isolated from certain types of infections related to biomedical implants, including central venous catheters, cerebrospinal fluid shunts, prosthetic heart valves, and ocular lens implants, was found to be *S. epidermidis* [10]. Bacteria colonize prosthetic implants as a biofilm, multiple layers of sessile cells that adhere to the implant surface as well as to each other. An additional problem of these infectious diseases is the increasing resistance of staphylococci to oxacillin and other antibiotics [5].

Biofilm formation by *S. epidermidis* is thought to be a two-step process; i.e., initial attachment of bacteria to a substrate surface, and bacterial accumulation by cell-cell adhesion using polysaccharide intercellular adhesin (PIA), which has linear β-1,6-linked glucosaminylglycans [25]. PIA is found to be mediated by the products of a gene locus comprising four intercellular adhesion (*ica*ADBC) genes, which are organized in an operon structure [18]. It was further demonstrated that both *ica*A and *ica*D mediate the synthesis of sugar oligomers *in vitro*, using UDP-*N*-acetylglucosamine as a substrate. This *N*-acetylglucosaminyltransferase activity, together with the activity of *ica*C, produces *in vitro* a product that is recognized by an antibody raised against PIA [15]. The presence of the *ica* operon and the biofilm formation are

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more common features in clinical isolates than in saprophytic isolates [2].

In this study, we examined the antibiotic multiresistance of *S. epidermidis* biofilms formed by the *ica* operon. To evaluate the potential contribution of biofilm production by the *ica* operon, we compared 50 strains isolated from patients with septicemic diseases and 124 skin isolates from healthy volunteers. The data indicated that the clinical isolates were different from the saprophytic isolates in terms of antibiotic susceptibility in association with the biofilm formed by the *ica* operon.

MATERIALS AND METHODS

Bacterial Strains

Total 174 *S. epidermidis* strains (124 skin strains obtained from healthy volunteers and 50 strains obtained from blood culture that caused septicemic diseases) were examined. Each strain was isolated from different people. Saprophytic strains were isolated from healthy people with wab-stab (Mast Diagnostica, Reinfeld, Germany), brought into the laboratory, and cultured on the blood agar plate.

Species Identification

For the identification of *Staphylococcus* species, the catalase test, coagulase test with staphytect plus-kit (Oxoid, Wesel, Germany), and API-Staph Ident System (BioMerieux, Nürtingen, Germany) were used.

Antibiotic Susceptibility Test

Antibiotic susceptibility was determined by agar disk diffusion (Kirby-Bauer method). The following antibiotics were tested: penicillin (10 U per disk), oxacillin (1 μg), gentamicin (10 μg), tetracycline (30 μg), erythromycin (15 μg), vancomycin (30 μg), chloramphenicol (30 μg), and clindamycin (2 μg). The strains that were resistant to three or more antibiotics were considered as multiresistant strains.

PCR Primers and Amplification

For hybridization, chromosomal DNA from *S. epidermidis* RP62A was amplified with primers of *ica* genes. For the detection of *ica* genes, the following primers were used: for the *ica*AD gene, primers icaA1 (GACCTCGAAGTCA-ATAGAGGT) and icaA2 (CCCAGTATAACGTTGGATACC); for the *ica*B gene, primers icaB1 (ATGGCTTAAAGCAC-ACGACGC) and icaB2 (TATCGGCATCTGGTGTGACAG); and for the *ica*C gene, primers icaC1 (ATAAACTTGAAT-TAGTGTATT) and icaC2 (ATATATAAAACTCTCTTAACA). The reaction was carried out in 25 μl total 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 the genes was used, including the following parameters: incubation at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min (denaturation), 55–58°C for 1 min (annealing), 72°C for 1 min (extension), and 72°C for 3 min after conclusion of the 30 cycles. After amplification, 10 μl of the PCR mixtures was analyzed by agarose gel electrophoresis (2% agarose in Tris-borate-EDTA).

Biofilm Assay

Bacteria were grown overnight in Tryptic soy broth (TSB; Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C. Cultures were then diluted in 1:200 ratio and incubated overnight in a tissue culture microtiter plate (Greiner, Nürtingen, Germany). Microtiter wells were washed three times with phosphate-buffered saline [PBS; 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 130 mM NaCl (pH 7.4)], dried in an inverted position, and stained for 10 min with 0.4% crystal violet solution [9]. The absorbance at 490 nm was determined. An OD_{490} of >0.120 was regarded as biofilm positive.

Isolation of Chromosomal DNA and Restriction Endonuclease Cleavage

The bacterial cells were grown in TSB medium overnight at 37°C. The overnight cultures were diluted in 1:100 ratio and grown to the mid-log phase in Luria-Bertani broth (Difco) supplemented with 1% glycine, at 37°C. Bacteria in 1.5 ml of the culture were harvested by centrifugation. The bacterial pellet was resuspended in 100 μl of 10 mM Tris-HCl (pH 7.5) containing 25% sucrose, 15 μl of 500 mM EDTA (pH 8.0), and 10 μl of lysostaphin (2 mg/ml) (Sigma, Deisenhofen, Germany), and incubated for 10 min at 37°C. After lysis of the bacterial cells, the chromosomal DNA was isolated by the standard procedures [29]. For digestion, DNA was incubated with *Eco*RI restriction enzyme (Gibco, Eggenstein, Germany) according to the recommendations of the manufacturer. After restriction endonuclease digestion, the DNA was analyzed by agarose gel electrophoresis (1% agarose in Tris-phosphate-EDTA).

Southern Hybridization

Gels were blotted on nylon membranes (Hybond-N^+, Amersham Life Science, Little Chalfont, England) by the standard methods [29]. Chromosomal DNA hybridization with each *ica*AD, B, and C were performed. Labeling of DNA probe and hybridization were performed by using the nonradioactive ECL direct nucleic acid-labeling and detection system (Amersham Life Science) according to the manufacturer's instructions. For hybridization, the hybridization buffer contained 0.5 M NaCl, and high-stringency washing steps were performed at 55°C in a buffer containing 0.1×standard saline citrate (SSC) and 0.4% sodium dodecyl sulfate. When a strain with all of the

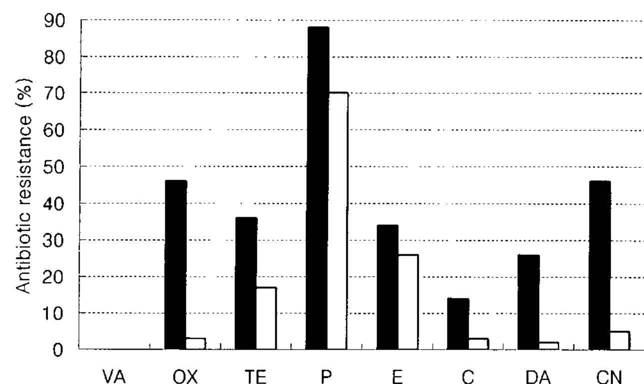

Fig. 1. Comparison of antibiotic resistance between clinical (■) and saprophytic (□) isolates of *S. epidermidis* strains.
Clinical isolates are the strains isolated from the blood culture of septicemic patients, and saprophytic isolates are the strains isolated from the skin of healthy persons. VA, vancomycin; OX, oxacillin; TE, tetracycline; P, penicillin; E, erythromycin; C, chloramphenicol; DA, clindamycin; CN, gentamicin.

ica genes was detected, the strain was considered as an *ica*-positive strain.

RESULTS

Antibiotic Susceptibility in Pathogenic and Nonpathogenic *S. epidermidis* Strains

The 174 *S. epidermidis* strains, including 50 clinical isolates obtained from blood cultures of patients and 124 saprophytic isolates, were tested for their antibiotic resistance. A significant difference between the blood culture isolates and saprophytic strains was observed: In the clinical isolates, 88% of the strains were penicillin resistant, 46% were oxacillin and gentamicin resistant, 34% were erythromycin resistant, 36% were tetracycline resistant, 26% were clindamycin resistant, and 14% were chloramphenicol resistant. On the other hand, the antibiotics resistances of the saprophytic strains were lower than the blood culture isolates. Among the antibiotics tested, the resistances to oxacillin, chloramphenicol, clindamycin, and gentamicin were drastically lower (Fig. 1).

Antibiotic multiresistance of these strains was analyzed. In the blood culture isolates, more antibiotic multiresistant strains were detectable than in the saprophytic isolates. As demonstrated in Table 1, 46% (23 of 50 strains) of the strains in the blood culture isolates were resistant to three or more antibiotics, whereas only 12% of the saprophytic isolates were resistant to three or more antibiotics. Moreover, the ratio of strains resistant to more than three antibiotics was relatively high in the blood culture isolates: In the blood culture isolates, 32% of the strains were resistant to five or six antibiotics, whereas almost none of the saprophytic strains were resistant to more than three antibiotics.

Table 2. Comparison of biofilm formation between the strains.

Type of isolates(n)	No. of isolates	
	Biofilm +	Biofilm –
Blood culture(50)	19	31
Saprophytic(124)	9	115

Biofilm formation was measured by a biofilm assay, as described in Materials and Methods.

Effect of Biofilm on the Antibiotic Resistance

To obtain information on biofilm formation of these strains, the strains were tested for their capacity to form biofilms on plastic material by quantitative adherence assay. The capacity of biofilm formation was different between the blood culture isolates and saprophytic isolates. As seen in Table 2, the biofilm formation of the blood culture isolates was more common than saprophytic isolates: 19 strains of 50 blood culture isolates produced biofilm, whereas only 9 strains of 124 saprophytic isolates formed biofilm.

To elucidate whether biofilm has any influence on antibiotic resistance, the data of the antibiotic multiresistance were analyzed in relation to the biofilm formation. As seen in Fig. 2, in both the blood culture isolates and the saprophytic isolates, biofilm-producing strains exhibited higher ratios of the antibiotic multiresistance than biofilm-nonproducing strains: In the blood culture isolates, 84% (16 of 19) of the biofilm-producing strains were antibiotic multiresistant, while only 22% (7 of 31) of the biofilm-nonproducing strains were antibiotic multiresistant. In the saprophytic isolates, the results were also similar to those in the blood culture isolates.

Correlation of the *ica* Gene Cluster for the Antibiotic Resistance

The strains were tested for the presence of the *ica* gene cluster by Southern hybridization of the chromosomal DNA. To elucidate whether the presence of the *ica* gene

Table 1. Characteristic of antibiotic multiresistance of the *S. epidermidis* strains tested.

Type of isolates(n)	No.(%) of resistant antibiotics						
	0	1	2	3	4	5	6
Blood culture(50)	6(12)	19(38)	2(4)	6(12)	1(2)	11(22)	5(10)
Saprophytic(124)	37(30)	45(36)	28(22)	12(10)	1(1)	1(1)	

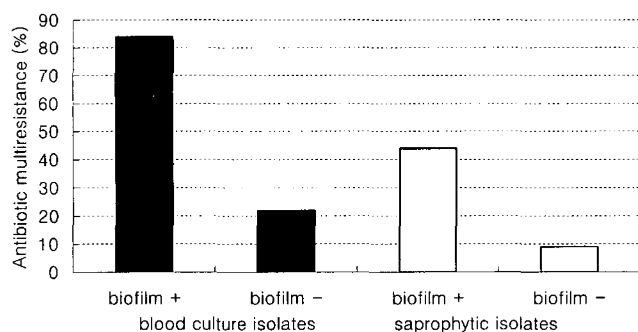

Fig. 2. Correlation of antibiotic multiresistance with the biofilm formation.
Antibiotic multiresistant strains were resistant to three or more antibiotics.

cluster was correlated with the antibiotic resistance, the results of the presence of the *ica* gene cluster were compared with the data of the antibiotic multiresistance.

As shown in Table 3, the presence of the *ica* gene cluster was found to be related with the biofilm formation. In the 19 biofilm-producing blood culture isolates, 18 strains possessed the *ica* gene cluster in chromosomal DNA, while this gene cluster was present only in 9 of 31 biofilm-nonproducing strains. In the saprophytic isolates, the gene was present in 7 of 9 biofilm-producing strains and in 15 of 115 biofilm-nonproducing strains.

A correlation between the *ica* gene cluster and the antibiotic multiresistance was found, and shown as summarized data in Fig. 3. In the biofilm-producing *ica*-positive strains, 89% (16 of 18) of the blood culture isolates and 57% (4 of 7) of the saprophytic isolates were antibiotic multiresistant, whereas no strains of the three *ica*-negative strains were antibiotic multiresistant. In the biofilm-nonproducing strains, 67% (6 of 9) of the *ica*-positive blood culture isolates, but only 7% (1 of 15) of the *ica*-positive saprophytic isolates, were antibiotic multiresistant. The ratios of the antibiotic multiresistance in the *ica*-negative strains was very low; 4% (1 of 22) in the blood culture isolates and 9% (9 of 100) in the saprophytic isolates.

Table 3. Detection of the *ica* gene cluster related with biofilm formation in the strains.

Type of isolates(n)	Biofilm +		Biofilm −	
	ica +	*ica* −	*ica* +	*ica* −
Blood culture(50)	18	1		22
Saprophytic(124)	7	2	15	100

DISCUSSION

It has been estimated that 65% of human nosocomial infections are biofilm-associated [25]. Oligosaccharide of bacteria is not generally considered to be a virulence factor. However, it has been suggested to play an indirect role in providing the attachment site and a crucial role in establishing the essential barrier function [11, 31]. *Staphylococcus* biofilms have been extensively studied in humans, and this pathogen has been considered to be significant in both device-associated infections and tissue infections such as pneumonia and osteomyelitis [4]. The prevalence of bovine staphylococcal mastitis ranges from 7% to 40% of all dairy cattle, and this infection is associated with bacterial biofilms [3]. *S. epidermidis* is an epidermal microflora of healthy humans; however, it has also been an important pathogen causing nosocomial infections [12, 23]. Many previous studies found that these infections are related with the use of indwelling medical devices [28]. Biofilm formation on polymer surfaces is thought to be the fundamental pathogenic mechanism of *S. epidermidis* infections [8]. This process is mediated with the *ica* operon, whose structure and function are very well known [2, 13, 15, 18, 25, 28, 39]. In addition, other proteins that contribute to biofilm formation have also been identified; for example, autolysin (AtlE), accumulation-associated proteins (AAP), biofilm-associated protein (Bap), and ClfA-related fibrinogen-binding protein (Fbe) [19, 21, 26, 30].

Bacterial cells in nosocomial infections exhibit increased resistance to antimicrobial agents. Our data in this study revealed that the enhanced antibiotic resistance of clinical

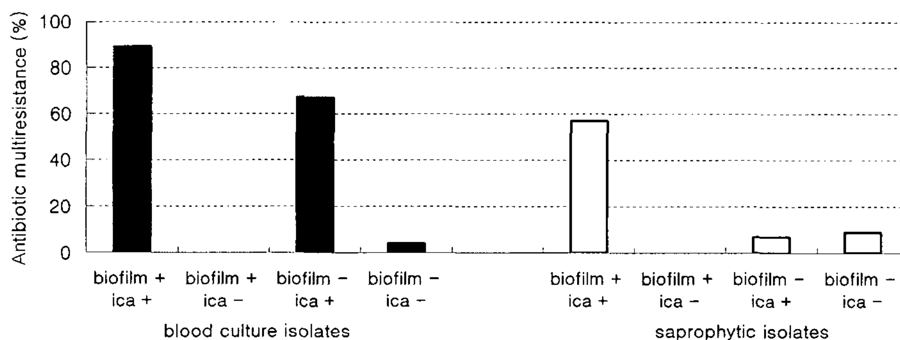

Fig. 3. Correlation of the *ica* gene cluster with antibiotic multiresistance.
Chromosomal DNAs isolated from the strains were digested with *Eco*RI and hybridized with *ica*-specific genes, as described in Materials and Methods.

isolates was a more common phenotype than mucosal isolates of *S. epidermidis*. In addition, the presence of the *ica* gene cluster and the biofilm formation of strains isolated from blood cultures of septicemic patients were more prevalent than those of strains from skin and mucosa. Moreover, the data revealed a strong correlation between the *ica* gene cluster and the biofilm formation. These results led us to a question of whether the enhanced antibiotic resistance was related with the biofilm formed by the *ica* gene cluster. In order to explore this question, we compared the results of the antibiotic resistance and the presence of the biofilm formed by the *ica* gene cluster, and found a strong correlation between the antibiotic multiresistance and the biofilm produced by the *ica* gene cluster (Fig. 3). Based on these results, it is highly likely that the biofilm produced by the *ica* gene cluster is an important factor for the increased antibiotic resistance of *S. epidermidis* strains.

Biofilm resistance may be multifactorial, although further experimentation is necessary. The production of an exopolysaccharide matrix may contribute to increase of cell survival by delaying antimicrobial penetration. The impact of transport limitation on biofilm survival has been analyzed in a number of studies [1, 22, 33, 37], and this antibiotic reisistance in biofilms has been confirmed to be due to bacterial growth and metabolic activity [24, 32, 35]. It has been proposed that slow-growing and non-growing bacteria considerably contribute to decrease biofilm susceptibility to antimicrobial agents. Oxygen availability is also postulated to contribute to antimicrobial resistance in biofilms, since the absence of oxygen was found to reduce the antimicrobial activity of some antibiotics [34, 38]. Additional studies are still needed to precisely elucidate the mechanism of how the biofilms produced by the *ica* gene cluster contribute to the antibiotic resistance.

Each gene and gene product contributing to this resistance could possibly be a target for the development of new chemotherapeutic agents. In the present study, the *ica* gene cluster was clearly characterized as the target gene to enhance antibiotic effects. Disabling of biofilm resistance may enhance the ability of existing antibiotics to clear infections involving biofilms that are refractory to current treatments. The expression of the *ica* gene cluster was found to undergo a phase variation. In previous studies, it was shown that IS*256* can be involved in phase variation in *S. epidermidis* [7, 40]. This phenotypic variation has been linked to increased resistance in several bacterial pathogens. Therefore, further experiment is required to elucidate the role of phase variation of biofilm formation in antibiotic resistance.

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