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Alcoholic ingredients in skin disinfectants increase biofilm expression of *Staphylococcus epidermidis*

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The pathogenesis of *Staphylococcus epidermidis* is correlated with biofilm formation. We investigated the effect of three common alcoholic skin disinfectants, ethanol, *n*-propanol and isopropanol, on the biofilm formation of 37 clinical, *icaADBC*-positive *S. epidermidis* isolates. In alcohol-supplemented media 18 strains displayed increased biofilm expression. Sixteen of 19 strains were generally incapable of biofilm formation. In three representative isolates, the increase in biofilm formation was paralleled by increased polysaccharide intercellular adhesin synthesis. Regarding the widespread use of alcoholic skin disinfectants, it is possible that the alcohol-inducible biofilm phenotype of *S. epidermidis* could add to the development of foreign body-related infections.

Introduction

In recent years *Staphylococcus epidermidis* has been isolated with increasing frequency as a causative pathogen of nosocomial infections, which are often foreign body related. The major pathogenic factor is the ability to form biofilms on polymeric surfaces.[1] Essential for cell accumulation is the expression of a polysaccharide intercellular adhesin (PIA), which mediates cell-to-cell adhesion and is synthesized by the *icaADBC* gene products.[1] Recently, it was shown that expression of *icaADBC* is dependent on RsbU, a positive regulator of σ^B, and that ethanol stress induces biofilm formation.[2,3]

Alcoholic skin disinfectants are frequently used, resulting in a high extent of bacterial elimination; however, small numbers of bacteria still survive on skin.[4] We therefore investigated the effect of the three most common alcoholic ingredients of skin disinfectants: ethanol, *n*-propanol and isopropanol, on biofilm formation of *S. epidermidis*.

Materials and methods

Bacterial strains, growth conditions and phenotypic characterization

S. epidermidis strains 1457 and 8400 were used as reference organisms.[5] One hundred and thirty-eight clinical isolates of *S. epidermidis* were sampled during a 12 month period from February 1998 to January 1999. The isolates were from blood cultures ($n = 56$), central venous or peritoneal dialysis catheters (74) and other relevant clinical specimens (eight). Isolates were identified using standard microbiological techniques.[5] Biofilm production was measured by a semi-quantitative adherence assay in trypticase soy broth (TSB_{BBL}; Becton Dickinson, Cockeysville, MD, USA).[3,6] Biofilm formation was classified into strongly biofilm positive ($OD_{570} \geqslant 1$), low grade biofilm-positive ($0.1 \leqslant OD_{570} < 1$) and biofilm negative ($OD_{570} < 0.1$). For analysis of the influence of disinfectants, TSB_{BBL} was supplemented with different concentrations of ethanol [1, 2, 4 or 6% (v/v)], *n*-propanol [0.5, 1, 2 or 4% (v/v)], isopropanol [1, 2, 4 or 6% (v/v)], benzalkonium chloride (0.1, 0.01 or 0.001 μg/mL) and chlorhexidine (0.1, 0.01 or 0.001 μg/mL), respectively. Increased biofilm formation due to the different alcohols was defined as at least OD_{570} 0.2 when the strain was primary biofilm negative, or at least doubling of the OD_{570} for low-grade biofilm-positive strains.

Quantification of PIA synthesis

Bacterial extracts and cell supernatants were prepared as described previously.[3] PIA concentrations were determined by a co-agglutination assay with PIA-specific antiserum.[6] Immunochemical variant PIA (PIAv), expressed

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by strain CNS161, was detected using an antiserum raised against *S. epidermidis* 7415, which expressed a polysaccharide not reactive with anti-PIA antiserum, absorbed with the isogenic biofilm-negative Tn*917* mutant 1457-M11.[5]

Genotypic characterization

Chromosomal DNA of *S. epidermidis* was prepared and amplification of DNA fragments was performed as described previously.[3,6] Amplification of a part of *icaB* was performed as described previously.[6] Oligonucleotides specific for *icaR* (icaR for 5′-ACTGGTAAGTCCGTCAAGT-3′) and *icaC* (icaC-H rev 5′-CAAGCACATACATAAGCCATAG-3′) of *S. epidermidis* (GenBank accession no. U43366) were synthesized by MWG Biotech (Munich, Germany).[7]

Pulsed-field gel electrophoresis (PFGE) was performed as described previously for analysis of clonal relationships.[2] Strains with identical PFGE patterns were only included for further analysis when they were isolated from different patients and not temporally related.

Results

RsbU, a positive regulator of the alternative sigma factor σ^B, positively controls biofilm formation in *S. epidermidis*.[3] Thereby, ethanol stress leads to increased PIA production and biofilm formation in the reference strains *S. epidermidis* 1457 and 8400.[3] To further investigate this phenotype, 138 clinical isolates of *S. epidermidis* were analysed for the presence of *icaADBC* by *icaB*-specific PCR, and the ability for biofilm formation in a standard biofilm assay. Sixty-eight strains (49%) were *icaB* positive; 70 strains (51%) displayed no *icaB* signal. Of the *icaB*-positive strains, 29 (43%) were strongly biofilm positive, 17 (25%) displayed low-grade biofilm expression and 22 (32%) were biofilm negative. Owing to limitations of photometers, an increase of biofilm formation in strongly biofilm-producing *S. epidermidis* strains could barely be detected.[3] We therefore further investigated 39 biofilm-negative and low-grade biofilm-producing strains. PFGE analysis of these strains revealed 33 different patterns (Table 1). PFGE patterns 2 and 11, and patterns 6 and 15, were represented by two and three strains, respectively. Two of the pattern 6 strains were excluded from further analysis because their isolation was temporally related, indicating nosocomial transmission.

Biofilm formation was analysed in TSB_{BBL} supplemented with different concentrations of ethanol, *n*-propanol and isopropanol. In 18 of 37 strains, increased biofilm formation was induced by ethanol, whereas 15 and 14 of these strains were inducible by *n*-propanol and isopropanol, respectively (Table 2). In the group containing low-grade biofilm-producing strains, 14 of 17 displayed increased biofilm formation, whereas only three strains were not inducible. The induction patterns of three representative clinical isolates are shown in the Figure (a–c). For these strains, expression of PIA was investigated using the optimum-inducing alcohol concentrations. For CNS27 and CNS156, increased cell-associated and supernatant PIA concentrations were detected (Figure). CNS161 did not react with the anti-PIA antiserum. Increased expression of PIAv was detected with absorbed antiserum raised against *S. epidermidis* 7415 (Figure). Using this antiserum, similar antigen concentrations were detected with strains CNS27 and CNS156, indicating that PIA and PIAv structures are closely related (Figure).

Sixteen of 20 biofilm-negative strains were not inducible by alcohol supplementation, indicating a general incapability of biofilm formation. To exclude functional genetic defects, *icaADBC* of these strains was amplified spanning *icaR* to *icaC*. No difference in size of the fragments was observed, indicating an intact *icaADBC* locus (data not shown). However, the possibility of point mutations cannot be excluded.

In media supplemented with increasing alcohol concentrations, the growth rate of the cells was decreased, whereas biofilm formation was increased for inducible *S. epidermidis* strains (data not shown). *n*-Propanol displayed the strongest effect, inhibiting cell growth totally at a concentration of 4%. In media supplemented with 6% ethanol or 6% isopropanol, some strains still displayed slow growth and a few strains showed increased biofilm formation (Figure).

Strains displaying increased biofilm formation were investigated for the influence of chlorhexidine and benzalkonium chloride on biofilm expression. For both compounds no positive influence on biofilm formation was observed at subinhibitory concentrations, while biofilm formation decreased in parallel with decrease in the growth rate (data not shown).

Discussion

S. epidermidis biofilm formation and PIA synthesis is quantitatively modulated by environmental factors.[1,3] The increasing biofilm formation due to ethanol supplementation by *S. epidermidis* reference strains suggests that this phenomenon could be of relevance for the development of nosocomial *S. epidermidis* infections in the clinical setting, where alcoholic skin disinfectants are routinely used. In the present study we characterized the effect of three alcohols, ethanol, *n*-propanol and isopropanol, on biofilm formation by 37 unrelated clinical isolates of *S. epidermidis*, which harbour the *icaADBC* gene locus and displayed no or low biofilm formation.[3]

A total 18 of 37 (49%) strains were inducible to increased biofilm formation by at least one of the alcohols. Increased biofilm formation was linked to increased production of PIA in strains CNS27 and CNS156. Interest-

Table 1. Phenotypic and genotypic properties of *S. epidermidis* strains investigated

Strain	PFGE pattern	*icaB* PCR	Biofilm in TSB	Inducible by		
				ethanol	*n*-propanol	isopropanol
CNS15	1	+	+	–	–	–
CNS23	2	+	+	+	+	+
CNS201	2	+	–	–	–	–
CNS27	3	+	–	+	+	+
CNS38	4	+	+	+	+	+
CNS39	5	+	+	+	–	–
CNS41	6	+	–	–	–	–
CNS42	7	+	+	+	+	+
CNS44	8	+	+	+	+	+
CNS75	9	+	–	–	–	–
CNS76	10	+	–	–	–	–
CNS84	11	+	–	–	–	–
CNS136	11	+	–	–	–	–
CNS85	12	+	+	+	+	–
CNS103	13	+	+	+	+	+
CNS112	14	+	+	–	–	–
CNS124	15	+	–	–	–	–
CNS126	15	+	–	–	–	–
CNS143	15	+	–	–	–	–
CNS127	16	+	–	–	–	–
CNS133	17	+	+	–	–	–
CNS137	18	+	–	–	–	–
CNS142	19	+	–	–	–	–
CNS146	20	+	–	–	–	–
CNS147	21	+	–	–	–	–
CNS148	22	+	+	+	+	+
CNS156	23	+	+	+	+	+
CNS158	24	+	–	+	+	+
CNS161	25	+	+	+	+	+
CNS163	26	+	–	+	–	–
CNS166	27	+	–	–	–	–
CNS168	28	+	–	+	+	+
CNS174	29	+	+	+	+	+
CNS185	30	+	+	+	+	+
CNS188	31	+	–	–	–	–
CNS189	32	+	+	+	–	–
CNS194	33	+	+	+	+	+

ingly, CNS161 turned out to be a strain expressing PIAv, an apparent immunochemical variant of PIA. Absorbed antisera raised against the PIAv-producing *S. epidermidis* 7415 was cross-reactive to PIA, indicating that both antigens are structurally related. PIAv expression was also stimulated by the different alcohols, indicating that expression of PIAv is regulated in a similar manner to PIA.[3]

In the group of primary low-grade biofilm-forming *S. epidermidis* strains, 82% displayed an inducible phenotype due to at least one of the alcohols tested. Surprisingly, only 20% of the primary biofilm-negative *icaADBC*-positive *S. epidermidis* displayed an increase in biofilm formation after alcohol supplementation, indicating a general incapability of these strains to form biofilm. No difference in size could be detected in PCR fragments spanning *icaRADBC* of all non-inducible strains, indicating an intact *icaADBC* locus. Apparently, insertions by insertion sequences in *icaADBC*[8,9] occur infrequently.

In addition to *icaADBC* there are at least three independent gene loci influencing PIA expression and biofilm

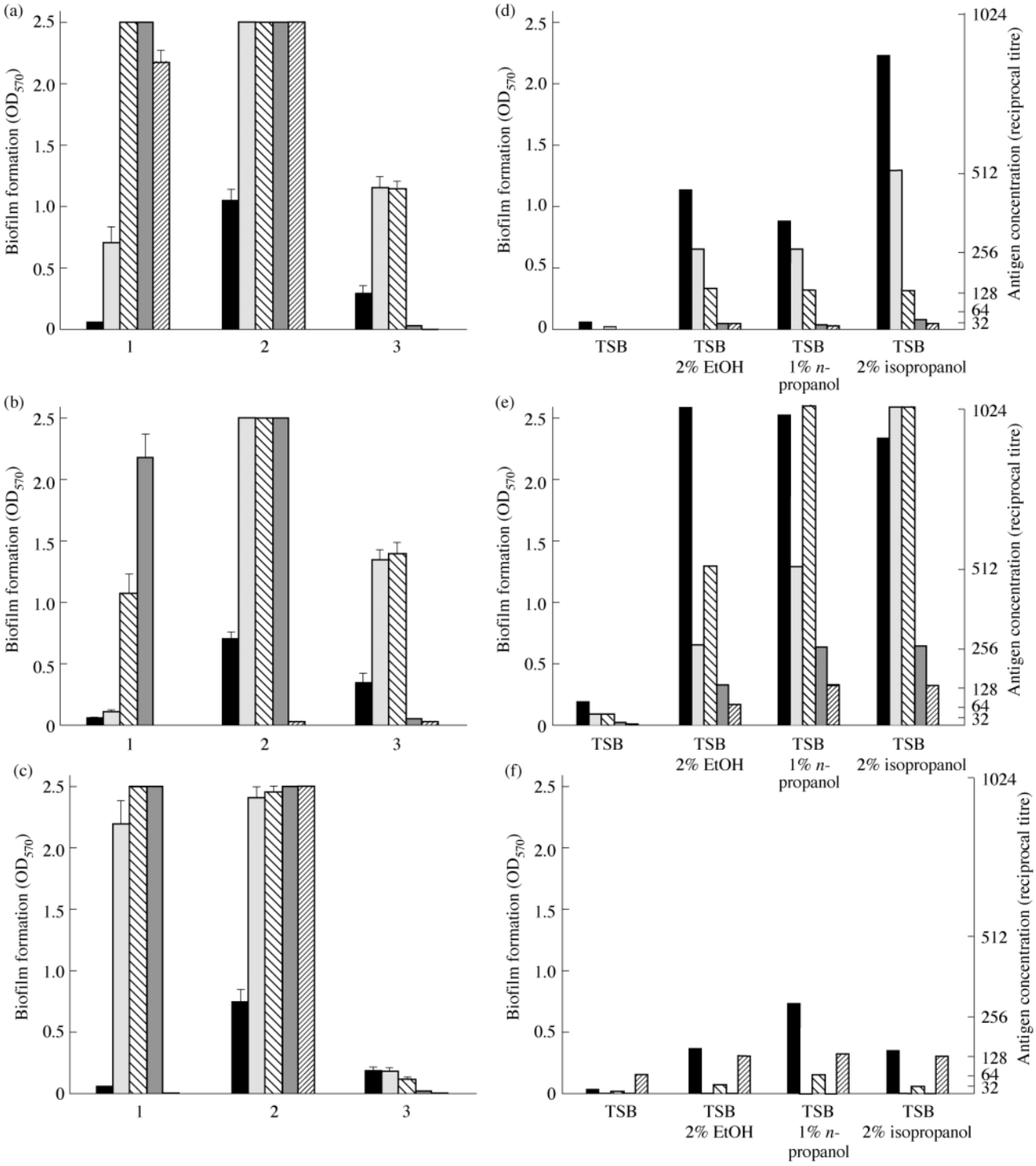

Figure. Induction of biofilm formation (a–c) in three representative *icaADBC*-positive clinical isolates in TSB_{BBL} supplemented with ethanol [EtOH (a); ■, TSB; □, 1%; ⧅, 2%; ■, 4%; ▨, 6%], *n*-propanol [*n*-prop.; (b); ■, TSB; □, 0.5%; ⧅, 1%; ■, 2%; ▨, 4%] and isopropanol [isoprop.; (c); ■, TSB; □, 1%; ⧅, 2%; ■, 4%; ▨, 6%]. The primary biofilm-negative strain 1 (CNS27) displayed strong biofilm induction by all three alcohols investigated (a–c). The primary biofilm-positive strain 2 (CNS156) is also strongly inducible by all tested alcohols even at high alcohol concentrations (a–c), whereas strain 3 (CNS161) displayed weaker induction only for ethanol and *n*-propanol (a, b). Additional induction of strain 3 was only detectable for lower alcohol concentrations in the media (a, b). Results of representative experiments are shown. Error bars indicate standard errors. PIA expression [(d–f); ■, biofilm; □, PIA cells; ⧅, PIAv cells; ■, PIA supernatant; ▨, PIAv supernatant] by *S. epidermidis* after induction of biofilm formation by ethanol, *n*-propanol and isopropanol. *S. epidermidis* strains CNS27 (d), CNS156 (e) and CNS161 (f) were grown in TSB_{BBL} or in TSB_{BBL} supplemented with the respective alcohol concentration as stated. Bacterial extracts and culture supernatants were prepared, and PIA and PIAv concentrations were determined by co-agglutination using specific antisera.

Table 2. Induction of biofilm formation in 37 *icaADBC*-positive, biofilm-negative or low-grade biofilm-positive clinical isolates by different alcohols

	Number	Inducible by			Not inducible (%)
		ethanol (%)	*n*-propanol (%)	isopropanol (%)	
Biofilm negative	20	4 (20)	3 (15)	3 (15)	16 (80)
Biofilm positive	17	14 (82.4)	12 (70.6)	11 (64.7)	3 (17.6)
Total	37	18 (48.6)	15 (40.5)	14 (37.8)	19 (51.4)

formation.[2] These gene loci could also be a target for mutation or deletion leading to a biofilm-negative phenotype. A *rsbU*-insertion mutant was biofilm-negative but could be induced by ethanol stimulation to produce biofilm and PIA.[3] Similar mutations could lead to the alcohol-dependent biofilm formation of biofilm-negative, *icaADBC*-positive clinical isolates.

The results with reference strains and primary biofilm-positive strains support the conclusion that a comparable percentage of the strongly biofilm-producing isolates display biofilm induction by alcohols. Therefore, >60% of the *icaADBC*-positive clinical isolates are predicted to be inducible in biofilm formation by alcohols in the environment.

For cutaneous antisepsis before the insertion of catheters and before surgery, as well as for surgical hand disinfection, alcoholic disinfectants are recommended.[4,10] In clinical use the tested alcohols are bactericidal. However, small numbers of bacteria remain viable after skin disinfection.[4] In addition, recolonization of the skin occurs, owing to the skin flora close to the disinfected area. Almost nothing is known about the diffusion and the following evaporation of the alcohols in the deeper layers of the skin. Therefore, the time course of an alcohol concentration gradient may reach conditions comparable to our test conditions for a relevant time period. It is reasonable to speculate that such conditions could confer a positive selective pressure on biofilm-positive *S. epidermidis* in deep skin layers and skin around the disinfection site, promoting the occurrence of medical device-related infections.

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