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In vitro effect of pH and ethanol on biofilm formation by clinical *ica*-positive *Staphylococcus epidermidis* strains

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Abstract - Biofilm production is an important step in the pathogenesis of *Staphylococcus epidermidis* associated biomaterial infections. *Staphylococcus epidermidis* strains isolated from dialysis fluid (n = 9) and needle cultures (n = 14) were phenotyped and genotyped for extracellular polysaccharide production and were examined for their ability to produce slime in a medium at various pH levels (3, 5, 7, 9 and 12) and with ethanol supplementation (0, 2, 5, 10 and 15%) using a semi-quantitative adherence assay. A total of 23 clinical *ica*ADBC positive *S. epidermidis,* one reference strain (*S. epidermidis* CIP 106510) used as positive control, and one *ica*ADBC negative strain (E21) were investigated. Qualitative biofilm production analysis revealed that 15 of the 23 *ica*ADBC positive strains (65.21%) produced slime on Congo Red agar plates. Quantitative biofilm was determined by measuring the optical density at 570 nm (OD_{570}). The results show that the slime production depended on the pH value of the medium and the ethanol concentration. At highly acidic (pH 3) and alkaline (pH 12) levels, the OD_{570} was lower, while at pH 7 the adhesion was moderate. In addition the cells adhered strongly with 2% ethanol than with the other concentrations. Our results suggest that pH and ethanol were stress factors that led to *S. epidermidis* biofilm formation and also play a possible role in the pathogenesis of biomaterial-related infections.

Key words: *Staphylococcus epidermidis*, biofilm, *ica* gene, Congo Red agar, pH, ethanol.

INTRODUCTION

Staphylococcus epidermidis is a major cause of medical device-associated infections. Coagulase-negative staphylococci (CoNS) have the ability to cause endovascular infections and are frequently associated with catheter-related bloodstream infections (Elliott *et al*., 1994). *Staphylococcus epidermidis* is an important infectious agent in patients with peritoneal dialysis catheters (Patrick *et al*., 1992). The virulence of *S. epidermidis*, such as its ability to produce gelatinase and slime, is dependent on host and environmental factors. As a member of human skin microflora, *S. epidermidis* can easily contaminate medical surfaces following surgical procedures (Mermel, 2000). Its adhesion to plastic materials and glass has been recognised as resulting from physico-chemical interactions between various elements (Bayer and Sloyer, 1990). It has been suggested that the ability of *S. epidermidis* to form biofilms on polymer surfaces greatly contributes to its spread (Peters *et al*., 1982; Christensen *et al*., 1985). This ability depends on the production of polysaccharide intercellular adhesion (PIA) encoded by the intercellular adhesion (*ica*) locus (McKenney *et al*., 1999; Von Eiff *et al*., 1999). Production of CoNS biofilm, which is composed of sessile bacterial cells embedded in a protective extracellular polysaccharide matrix, is widely considered to be an important pathogenic determinant in prosthetic device-related infections. The initial attachment of staphylococcal cells on a biomaterial is followed by bacterial accumulation in multiple layers, resulting in the formation of a mature biofilm (Götz, 2002) which is resistant to physical forces produced by blood flow and the washing action of saliva. Some studies have reported that this slime renders *S. epidermidis* highly resistant to antibiotics and host defences (Costerton *et al*., 1999).

It has been reported that in *S. epidermidis* the formation of a biofilm can be induced by conditions that are potentially toxic for the bacterial cell (Rachid *et al*., 2000a). Moreover, the characteristics of the suspension medium are considered to be significant factors in the microbial adhesion to the substrate. Alcohol-based skin disinfectants are frequently used, resulting in a high extent of bacterial elimination (Lawrence and Turner, 2005). Recently, ethanol washings were shown to promote biofilm formation (Mack *et al*., 2000; Knobloch *et al*., 2001).

The aims of this study was to evaluate the capacity of several *ica*ADBC positive strains of *S. epidermidis* isolated from dialysis fluid and needle cultures to producing slime at various pH levels and ethanol concentrations in polystyrene microtiter plates.

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MATERIALS AND METHODS

Bacterial strains and biochemical characterisation. The *S. epidermidis* strains (n = 23) used in this study, listed in Table 1, were isolated from dialysis fluid (n = 9) and needle cultures (n = 14) in one dialysis service in central Tunisia. In addition *S. epidermidis* CIP 106510 (Pasteur Institute Collection, Paris, France) was used as positive slime-producing and the non-producer strain E21, which lacked the *ica*ADBC gene, was used as negative control. Presumptive coagulase-negative strain identification was achieved by colony morphology, Gram staining, catalase positive and coagulase negative testing, and DNase analyses. Species identification was performed using the Api ID32 Staph system (bioMérieux, Marcy l'Étoile, France) according to the manufacturer's specifications. Results were read using an automated microbiological mini-Api (bioMérieux).

Phenotypic characterisation of bacteria-producing slime. Qualitative detection of the slime formation was studied by culturing the strains on Congo Red agar (CRA) plates, as previously described (Freeman *et al*., 1989). The strains were inoculated on CRA plates (36 g saccharose with 0.8 g Congo red in one litre of Brain Heart Infusion agar), incubated at 37 °C for 24 h under aerobic conditions, and followed overnight at room temperature (Vasudevan *et al*., 2003; Zmantar *et al*., 2006). The slime-positive variants appeared as rough, reddish-black colonies on the CRA plates, while the slime-negative strains developed smooth red colonies.

Detection of the *ica*ADBC locus. The presence of the entire *ica*ADBC gene was detected by PCR, using the forward and reverse primers previously described (McKenney *et al*., 1999). *Staphylococcus epidermidis* strains were grown overnight at 37 °C on inexpensive blood agar plates. One colony was suspended in one millilitre LB broth (Sigma, Aldrich, France) and cultured at 37 °C for 24 h. Chromosomal DNA was extracted using a Wizard Genomic purification Kit (Promega, Madison, USA.). The concentration of purified DNA was adjusted at 50 ng/µl using spectroscopy (Ultraspec 2100 pro, Amersham Biosciences, Europe GmbH, France).

The forward primer used for the *ica*ADBC had the following sequence: 5'-TGCACTCAATGAGGGAATCA-3', corresponding to nucleotides 409 to 428 in the *ica*A gene; the reverse primer had the following sequence: 5'-AATCACTACCGGAAACAGCG-3' which was complementary to nucleotides 3114 to 3133 in the *ica*C gene. PCR was performed in a Gene Amp PCR System 9700 (Applied Biosystems Int., USA). A typical PCR (25 µl) contained 1 µM

TABLE 1 - Relationships between biotype, the presence of the *ica* operon in *Staphylococcus epidermidis* strains, CRA phenotype and OD570

Strains	Biotype of strains	Presence of *ica* ADBC	Phenotype of strains (CRA)	Production of slime (CRA)	*OD570
CIP 106510	376010200	*ica* ADBC+	very black (vb)	Producer	++++
E21	366032200	*ica* ADBC-	very red (vr)	Non producer	–
E9	166050210	*ica* ADBC+	black (b)	Producer	++
S56	167032200	*ica* ADBC+	very black (vb)	Producer	++++
E18	366020200	*ica* ADBC+	bordeaux (brd)	Non producer	++
E7		*ica* ADBC+	black (b)	Producer	++
E24		*ica* ADBC+	very black (vb)	Producer	++
S22	366030210	*ica* ADBC+	very red (vr)	Non producer	–
S9		*ica* ADBC+	very black (vb)	Producer	++
S21	366032200	*ica* ADBC+	very black (vb)	Producer	++
E5	366032210	*ica* ADBC+	black (b)	Producer	++
S15		*ica* ADBC+	almost black (ab)	Producer	++
S16		*ica* ADBC+	very black (vb)	Producer	++
S43		*ica* ADBC+	very black (vb)	Producer	++
E11	366050210	*ica* ADBC+	bordeaux (brd)	Non producer	++
S33	367030210	*ica* ADBC+	bordeaux (brd)	Non producer	–
S59		*ica* ADBC+	bordeaux (brd)	Non producer	–
E15	376010200	*ica* ADBC+	very black (vb)	Producer	++++
E4	376032200	*ica* ADBC+	bordeaux (brd)	Non producer	++
E6		*ica* ADBC+	bordeaux (brd)	Non producer	++
S2		*ica* ADBC+	bordeaux (brd)	Non producer	++
S35		*ica* ADBC+	black (b)	Producer	++
S26		*ica* ADBC+	very black (vb)	Producer	++++
S40		*ica* ADBC+	very black (vb)	Producer	++++
S48		*ica* ADBC+	very black (vb)	Producer	–

(++++): highly biofilm-positive, (++): low-grade biofilm formation, and (-): biofilm negative.

forward and reverse primers, a dNTP mix (100 µM each of dATP, dCTP, dGTP and dTTP), 1 U of GO *Taq* DNA polymerase (Promega), 5 µl green Go *Taq* buffer (5X), and a DNA template (50 ng). The primers were designed to amplify a gene product of 2.7 kb encompassing a region of the *ica*ADBC genes of the staphylococcal *ica* locus. PCR conditions included initial denaturation (94 °C for 5 min), 30 denaturation cycles (95 °C for 30 s), annealing (52 °C for 30 s), extension (72 °C for 2.5 min), followed by a final extension (72 °C for 10 min) at the end of cycling. The PCR products (5 µl) were analysed on 0.7% agarose gel stained with ethidium bromide (0.5 µg/ml), viewed under UV transillumination and photographed using a Gel Doc XR apparatus (Bio-Rad, California, USA).

Adherence assay to measure biofilm production by *Staphylococcus epidermidis* cells as a function of pH value and ethanol concentration. Biofilm production by *S. epidermidis* strains grown in Trypticase Soy broth (TSB, Bio-Rad, France) was determined using a semi-quantitative adherence assay on 96-well tissue culture plates (Nunc, Roskilde, Denmark), as described previously (Christensen *et al*., 1985; Mack *et al*., 2001) with some modifications.

Following overnight incubation at 37 °C, the optical density at 600 nm (OD_{600}) of the bacteria was measured. An overnight culture grown in TSB at 37 °C was diluted to 1:100 in TSB with 2% (w/v) glucose to maximise *ica* operon induction, as reported elsewhere (Rachid *et al*., 2000a). A total of 200 µl of these cell suspensions was transferred in a U-bottomed 96-well microtiter plate (Nunc, Roskilde, Denmark). Each strain was tested in triplicate. Wells with sterile TSB alone were served as controls. *Staphylococcus epidermidis* CIP 106510 was used as the positive control and one *ica*ADBC negative strain (E21) as the negative control.

The plates were incubated aerobically at 37 °C for 24 h. The cultures were removed and the microtiter wells were washed twice with phosphate-buffered saline (7 mM Na_2HPO_4, 3 mM NaH_2PO_4 and 130 mM NaCl at pH 7.4) to remove non-adherent cells and were dried in an inverted position. Adherent bacteria were fixed with 95% ethanol and stained with 100 µl of 1% crystal violet (Merck, France) for 5 min. The excess stain was rinsed and poured off and the wells were washed three times with 300 µl of sterile distilled water. The water was then cleared and the microplates were air-dried. The optical density of each well was measured at 570 nm (OD_{570}) using an automated Multiskan reader (GIO. DE VITA E C, Rome, Italy). Biofilm formation was interpreted as highly positive ($OD_{570} \geq 1$), low-grade positive ($0.1 \leq OD_{570} < 1$), or negative ($OD_{570} < 0.1$). To analyse the degree of biofilm formation, the pH of the medium was adjusted (3, 5, 7, 9 and 12) and the TSB was supplemented with increasing concentrations of ethanol (2, 5, 10 and 15%, v/v). Each test was done in triplicate.

Increased biofilm formation caused by the effect of pH and alcohol supplementation was defined as being at least $OD_{570} = 0.2$ when the strain was primary-negative, or at least double OD_{570} when the strain was low-grade positive, as previously described (Knobloch *et al*., 2001).

Statistical analysis. Each analysis was performed using the SPSS 13.0 statistics package for Windows. The differences in the degree of biofilm formation were examined by the Friedman test, followed by the Wilcoxon signed ranks test. P-values < 0.05 were considered significant.

RESULTS

Detection of slime-producing *Staphylococcus epidermidis* strains

The PCR technique was applied to the staphylococcal strains. As reported in Fig. 1 and Table 1, a total of 23 *ica*ADBC positive strains of *S. epidermidis* were isolated from dialysis fluid (n = 9) and needle cultures (n = 14) and were investigated. The phenotypic slime production of each strain was assessed by means of cultures on CRA plates (Table 1). Results were interpreted as described elsewhere (Arciola *et al*., 2002). Among the 23 clinical isolates, 15 *S. epidermidis* strains (65.21%) produced slime, displaying black or darkened colonies, while the remaining eight strains were non producer, forming red or bordeaux-coloured colonies.

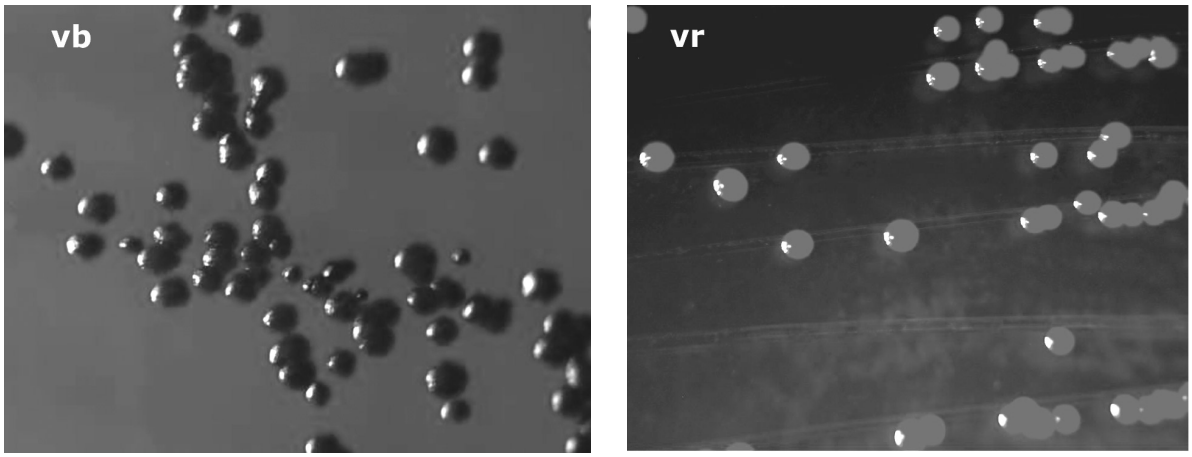

FIG. 1 - Colourimetric scale for colony analysis of slime production by *Staphylococcus epidermidis* using Congo Red agar assay (G8x). Left: slime producing strain, vb: very black; right: non producing strain, vr: very red.

Biofilm formation by *Staphylococcus epidermidis* at various pH levels

Each of the 23 *ica*ADBC positive strains (Fig. 2) was incubated in microplates with TSB at different pH levels. Table 2 presents the results of the OD_{570} at the different pH values. At pH 7, five strains (CIP 106510, S26, S40, E15 and S56) were highly biofilm-positive ($OD_{570} \geq 1$), fifteen strains showed low-grade biofilm formation ($0.1 < OD_{570} < 1$), and five were biofilm-negative (S22, S33, S59, S48 and E21). In addition, we observed that at an acidic pH level (pH 3), none of the tested strains produced slime ($OD_{570} < 0.1$). Interestingly, the OD_{570} at a high alkalinic level (pH 12) decreased for most of the tested strains compared to that observed at pH 7. Furthermore, at pH 5, the biofilm formation decreased in the reference strains, in all of the highly biofilm-positive strains (S26, S40, E15 and S56) and 4 of the 15 low-grade biofilm strains. At pH 5, an increase in biofilm formation was observed in strains S33 and S48. On the other hand, at pH 9, the reference strain as well as 11 of the 23 strains displayed an increase in biofilm formation, while four strains (E24, S9, S15 and E15) showed a decrease in biofilm formation. The remaining eight strains were not inducible.

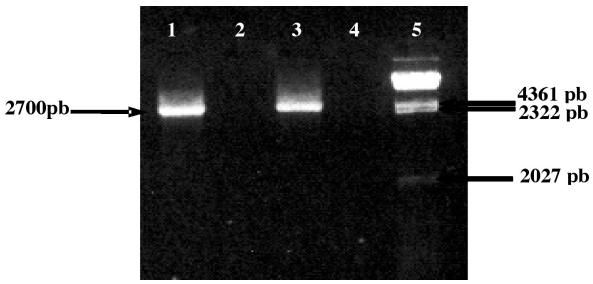

FIG. 2 - Agarose gel electrophoresis of PCR amplification of icaADBC locus. Lanes 1 to 4, PCR amplicons obtained with DNA of *Staphylococcus epidermidis*: line 1 refers to S56; line 2 to E21; line 3 to E24 and line 4 to the negative control. Line 5 refers to the HindIII DNA molecular size marker.

Effect of ethanol on *Staphylococcus epidermidis* biofilm formation

As is reported in Table 3, in the standard medium (without ethanol) 18 of the 23 strains under study were low-grade biofilm-positive ($0.1 < OD_{570} < 1$) and the strain E21 was a non-producer ($OD_{570} < 0.1$). Analysis of the data obtained with ethanol showed that a strong biofilm formation was induced with 2% ethanol in CIP 106510, S26, S40,

TABLE 2 - Biofilm formation by *Staphylococcus epidermidis* at different pH value

Strains	Biofilm formation (OD_{570})*				
	pH 3 (Mean value ± SD**)	pH 5 (Mean value ± SD)	pH 7 (Mean value ± SD)	pH 9 (Mean value ± SD)	pH 12 (Mean value ± SD)
CIP 106510	0.046 ± 0.001	1.121 ± 0.031	1.545 ± 0.059	1.837 ± 0.027	0.170 ± 0.343
E21	0.045 ± 0.004	0.090 ± 0.168	0.052 ± 0.038	0.024 ± 0.148	0.013 ± 0.085
E18	0.023 ± 0.018	0.268 ± 0.115	0.145 ± 0.097	0.134 ± 0.024	0.084 ± 0.056
E24	0.015 ± 0.011	0.277 ± 0.112	0.840 ± 0.033	0.261 ± 0.125	0.033 ± 0.114
S22	0.014 ± 0.016	0.004 ± 0.002	0.021 ± 0.009	0.022 ± 0.013	0.012 ± 0.006
S9	0.039 ± 0.022	0.120 ± 0.021	0.762 ± 0.07	0.027 ± 0.028	0.118 ± 0.024
S21	0.024 ± 0.015	0.087 ± 0.059	0.297 ± 0.032	0.576 ± 0.134	0.037 ± 0.198
E5	0.023 ± 0.018	0.225 ± 0.03	0.250 ± 0.185	0.167 ± 0.013	0.182 ± 0.037
S15	0.008 ± 0.004	0.190 ± 0.129	0.53 ± 0.15	0.149 ± 0.094	0.011 ± 0.134
E9	0.01 ± 0.007	0.023 ± 0.015	0.157 ± 0.08	0.177 ± 0.023	0.074 ± 0.078
S43	0.028 ± 0.013	0.155 ± 0.045	0.111 ± 0.048	0.112 ± 0.053	0.085 ± 0.035
E11	0.032 ± 0.011	0.261 ± 0.042	0.152 ± 0.038	0.318 ± 0.076	0.132 ± 0.151
S33	0.028 ± 0.015	0.235 ± 0.1	0.057 ± 0.018	0.236 ± 0.149	0.064 ± 0.109
S59	0.066 ± 0.056	0.048 ± 0.022	0.095 ± 0.066	0.288 ± 0.078	0.103 ± 0.067
E4	0.009 ± 0.002	0.409 ± 0.225	0.321 ± 0.092	0.385 ± 0.138	0.045 ± 0.157
S2	0.02 ± 0.011	0.160 ± 0.07	0.176 ± 0.086	0.445 ± 0.15	0.169 ± 0.091
S26	0.013 ± 0.007	0.868 ± 0.057	1.791 ± 0.173	2.548 ± 0.224	0.015 ± 0.005
S35	0.039 ± 0.008	0.247 ± 0.059	0.192 ± 0.037	0.197 ± 0.156	0.164 ± 0.083
E6	0.015 ± 0.011	0.303 ± 0.182	0.245 ± 0.002	0.387 ± 0.143	0.055 ± 0.127
S48	0.037 ± 0.012	0.350 ± 0.027	0.094 ± 0.04	1.709 ± 0.428	0.130 ± 0.273
S40	0.011 ± 0.01	0.268 ± 0.142	1.006 ± 0.159	1.219 ± 0.543	0.039 ± 0.483
E15	0.013 ± 0.003	0.364 ± 0.029	1.297 ± 0.246	1.086 ± 0.05	0.048 ± 0.056
E7	0.016 ± 0.0005	0.565 ± 0.177	0.459 ± 0.244	2.071 ± 0.444	0.209 ± 0.239
S16	0.02 ± 0.007	0.454 ± 0.11	0.326 ± 0.185	1.149 ± 0.125	0.117 ± 0.075
S56	0.025 ± 0.004	0.434 ± 0.06	1.058 ± 0.128	1.283 ± 0.129	0.074 ± 0.066

* $OD_{570} \geq 1$: highly biofilm-positive, $0.1 < OD_{570} < 1$: low-grade biofilm formation, and $OD_{570} < 0.1$ biofilm-negative.
** Standard deviation.

E15 and S56 (Table 3). In the group containing low-grade biofilm-producing strains, 7 out of 16 displayed increased biofilm formation, while 9 out of 16 strains were not inducible with ethanol supplementation. It is interesting to note that biofilm formation was promoted by 2% ethanol when S22, S33 and S48 were used. Indeed, in media supplemented with increasing ethanol concentrations (5, 10 and 15%), the growth rate of the *S. epidermidis* significantly decreased (OD_{570} < 0.1). At high levels of ethanol, bacterial growth was impaired.

DISCUSSION

In recent years, *S epidermidis* has emerged as a common cause of nosocomial infections associated with the use of intravenous catheters (Rupp and Archer, 1994). Microbial biofilms may pose a problem for persons requiring indwelling medical devices.

Twenty-three strains of *ica*ADBC *S. epidermidis* were isolated from dialysis fluid and needle cultures and investigated (Table 1). Fifteen strains were biofilm positive, forming reddish-black colonies on CRA plates, and eight were biofilm negative (red colonies). At pH 7, five strains (CIP 106510, S26, S40, E15 and S56) were categorized as highly biofilm-positive (OD_{570} ≥ 1), fifteen as low-grade (0.1 ≤ OD_{570} < 1), and four (S22, S33, S59 and S48) as biofilm-negative (Table 2). The negative control (E21) produced no slime (OD_{570} < 0.1).

At an acidic pH level (pH 3), no slime was produced by the tested strains (OD_{570} < 0.1). On the other hand, at pH 5, a decrease in biofilm formation was observed in four biofilm-positive reference strains (S26, S40, E15 and S56) and in 4 of the 15 low-grade biofilm strains, and was induced in two strains (S33 and S48). It has been demonstrated that the adherence of *S. aureus* cells is inhibited at acidic pH levels and enhanced by alkaline pH values. This may also be due to the lower pH level of these media (Memple et *al*., 1998).

In many biofilm-forming bacteria, the differentiation of planktonic cells into sessile, exopolysaccharide producing bacteria is associated with the environmental stress factors (Costerton et *al*., 1999, Shapiro 1998).

At the highly alkalinic pH 12, the OD_{570} decreased for most of the strains tested. At pH 9, the reference strain and 11 *S. epidermidis* showed increased biofilm formation; whereas four strains (E24, S9, S15 and E15) showed decreased biofilm formation and eight strains were not inducible as presented in Table 2. Statistical analysis revealed a significant difference between the OD_{570} obtained at pH 7-pH 3, at pH 7-pH 9 as well as at pH 7-

TABLE 3 - Biofilm formation by *Staphylococcus epidermidis* at different alcohol supplementation

Strains	Biofilm formation (OD_{570})*				
	Alcools 0% (Mean value ± SD**)	Alcools 2% (Mean value ± SD)	Alcools 5% (Mean value ± SD)	Alcools 10% (Mean value ± SD)	Alcools 15% (Mean value ± SD)
CIP 106510	1.786 ± 0.072	1.973 ± 0.067	0.657 ± 0.492	0.248 ± 0.145	0.106 ± 0.088
E21	0.036 ± 0.095	0.175 ± 0.108	0.090 ± 0.075	0.092 ± 0.090	0.079 ± 0.024
E18	0.260 ± 0.099	0.353 ± 0.129	0.262 ± 0.046	0.049 ± 0.046	0.040 ± 0.050
E24	0.778 ± 0.146	0.846 ± 0.249	0.388 ± 0.347	0.146 ± 0.080	0.070 ± 0.068
S22	0.063 ± 0.054	0.536 ± 0.014	0.066 ± 0.009	0.020 ± 0.014	0.040 ± 0.051
S9	0.931 ± 0.063	0.825 ± 0.281	0.909 ± 0.069	0.267 ± 0.204	0.044 ± 0.047
S21	0.715 ± 0.223	1.571 ± 0.496	0.492 ± 0.113	0.707 ± 0.571	0.020 ± 0.025
E5	0.327 ± 0.156	0.818 ± 0.075	0.381 ± 0.301	0.088 ± 0.064	0.035 ± 0.049
S15	0.481 ± 0.201	0.575 ± 0.070	0.159 ± 0.045	0.372 ± 0.123	0.074 ± 0.098
E9	0.316 ± 0.247	0.404 ± 0.095	0.285 ± 0.268	0.060 ± 0.013	0.077 ± 0.062
S43	0.122 ± 0.253	0.394 ± 0.300	0.192 ± 0.022	0.230 ± 0.229	0.054 ± 0.046
E11	0.242 ± 0.180	0.587 ± 0.020	0.422 ± 0.027	0.052 ± 0.027	0.029 ± 0.044
S33	0.022 ± 0.187	0.470 ± 0.374	0.304 ± 0.339	0.072 ± 0.029	0.036 ± 0.046
S59	0.106 ± 0.159	0.582 ± 0.186	0.631 ± 0.278	0.073 ± 0.067	0.023 ± 0.024
E4	0.426 ± 0.396	0.698 ± 0.175	0.286 ± 0.024	0.042 ± 0.061	0.036 ± 0.017
S2	0.164 ± 0.084	0.249 ± 0.073	0.030 ± 0.002	0.155 ± 0.047	0.042 ± 0.037
S26	1.495 ± 0.425	1.939 ± 0.014	0.194 ± 0.060	0.053 ± 0.018	0.022 ± 0.014
S35	0.420 ± 0.294	0.482 ± 0.205	0.429 ± 0.289	0.211 ± 0.070	0.063 ± 0.138
E6	0.298 ± 0.279	0.258 ± 0.194	0.226 ± 0.344	0.296 ± 0.054	0.041 ± 0.011
S48	0.091 ± 0.050	0.324 ± 0.017	0.102 ± 0.038	0.132 ± 0.075	0.042 ± 0.039
S40	1.040 ± 0.065	1.821 ± 0.036	0.158 ± 0.161	0.169 ± 0.115	0.037 ± 0.056
E15	1.188 ± 0.558	1.772 ± 0.197	1.414 ± 0.006	0.115 ± 0.017	0.052 ± 0.041
E7	0.773 ± 0.311	0.656 ± 0.310	0.989 ± 0.957	1.985 ± 0.356	0.067 ± 0.029
S16	0.304 ± 0.204	0.623 ± 0.112	0.310 ± 0.182	0.041 ± 0.013	0.033 ± 0.026
S56	1.375 ± 0.091	1.957 ± 0.151	0.491 ± 0.298	0.257 ± 0.051	0.030 ± 0.011

* OD_{570} ≥ 1: highly biofilm-positive, 0.1 < OD_{570} < 1: low-grade biofilm formation, and OD_{570} < 0.1 biofilm-negative.
** Standard deviation.

pH 12 ($P < 0.05$). However, the difference observed between the OD_{570} at pH 7 and that pH 5 was not significant ($P = 0.549$).

The results are in agreement with those of White et *al*., (1978) suggesting that the pH of the medium is an important factor in the induction of the *ica*ADBC gene. Moreover, pH has a marked influence on enzyme activity. Each enzyme has an optimal pH, and there is decreased activity on each side of this pH (White et *al*., 1978). The formation of a biofilm can be induced by conditions that are potentially toxic for the bacterial cell, and they confirm previous observations on biofilm activation by high osmolarity, detergents, urea, ethanol, and oxidative stress (Rachid et *al*. 2000a, b).

As the *S. epidermidis* cells used in this study were relatively hydrophobic, the low-grade *S. epidermidis* biofilm observed for some strains at alkaline pH 12 may be considered as a result of the strong hydrophobic attraction between the cells and a possible weak repulsion between the hydrophobic cells and the hydrophilic substrate. On the other hand, because the negative surface charge decreases at a low pH, the aggregation and low biofilm formation at the acidic pH may be partly attributed to a reduction of cell-cell repulsion. However, this phenomenon could be explained by the fact that the electrostatic properties of polystyrene surfaces may be influenced by pH value (Meinders et *al*., 1994).

Analysis of the data obtained with increasing ethanol concentrations showed that five strains (CIP106510, S26, S40, E15 and S56) cultured in TSB supplemented with 2 % ethanol (v/v) were strong biofilm producers. In the group containing the low-grade biofilm-producing strains, 7 out of 16 displayed increased biofilm formation, while 9 failed to produce biofilm. Interestingly, three strains (S22, S33 and S48) were induced by 2 % ethanol to become producing strains. The increase in biofilm formation due to the ethanol supplementation suggests that this phenomenon could be of relevance in the development of nosocomial *S. epidermidis* infections in the clinical setting, where alcohol-based skin disinfectants are routinely used. This data has been echoed in a later study in which the capacity to form biofilm was shown to depend on environmental conditions, such as stress or sub-inhibitory antibiotic concentrations (Conlon et *al*., 2002).

We also observed that biofilm formation was induced in the *ica*ADBC negative strain in the medium supplemented with 2% ethanol (E21). This result is in agreement with an earlier study which showed that biofilm production was also induced in a number of isolates lacking the *ica* gene (Knobloch et *al*., 2001). Furthermore, we found that the growth rate of the cells decreased significantly ($OD_{570} < 0.1$) after addition in the plate of 5 %, 10 % and 15 % ethanol.

In a recent report, Knobloch et *al*. (2001) suggested that alcoholic ingredients in skin disinfectants have the potential to increase biofilm formation in clinical isolates of *S. epidermidis*. Other research has shown that only a small proportion of the *ica*-positive *S. epidermidis* strains implicated in device-related infections produced biofilm under standard growth conditions. However, biofilm formation was induced when grown under stress-induced conditions (pH and ethanol) or in the presence of sub-inhibitory tetracycline (Fitzpatrick et *al*., 2002). In clinical use, the tested alcohols are bactericidal, yet small numbers of bacteria do remain viable following skin disinfection. In addition Rachid et *al*. 2000a, b demonstrated that ethanol, detergent, oxidative stress induced biofilm formation. Alcoholic disinfectants are currently recommended to counter cutaneous antisepsis prior to the insertion of catheters (Pearson, 1996). Generally speaking, there is a wide range of variations in the adherence and biofilm formation capacity of clinical *S. epidermidis* strains. However, no relationship has been found between the strain's surface hydrophobicity and the extent of the initial binding to hydrophilic or hydrophobic substrates (Cerca et *al*., 2005). In addition the anaerobic environment stimulates PIA production in both *S. aureus* and *S. epidermidis*.

Our study also confirms the many variations in biofilm formation found in clinical *S. epidermidis* strains. Numerous other studies have suggested that hydrophobic interactions contribute to the initial binding of pathogens to tissues (Doyle, 2000).

In conclusion, pH and ethanol stress induced biofilm formation, thus the use of effective skin disinfection is essential to reduce catheter-related sepsis. Furthermore, environmental conditions may also confer a positive selective pressure on biofilm-positive *S. epidermidis*, promoting the occurrence of medical device-related infections.

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