

# Slime production of *Staphylococcus epidermidis*

## Increased bacterial adherence and accumulation onto pure titanium

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In an in vitro study using *Staphylococcus epidermidis* RP 62 A, a slime-producing strain and its isogenic slime-negative mutant M7, we demonstrated that both strains adhere to pure titanium discs with significantly higher colony counts for the slime-producing strain. The colony count was dependent on temperature, time and strain. Prolonged incubation time (24 h) under growth conditions leads to higher colo-

ny counts for the slime-producing strain RP 62 A. As the slime-negative mutant M7 can adhere to, but not form multiple layers on metallic surfaces, increase of incubation time does not produce higher colony counts on the metallic surface. We conclude that slime production is important for adherence and subsequent accumulation of *S. epidermidis* onto pure titanium discs in vitro.

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Submitted 97-07-10. Accepted 98-05-05

*Staphylococcus epidermidis* (SE) is now recognized as a major pathogen involved in infections of medical implants (Peters et al. 1982, Sugarmann and Young 1989, Archer 1990, Peters et al. 1990). SE is occasionally isolated from periprosthetic samples from so-called "aseptically" loosened prosthetic joints (Barth et al. 1989, Perdreau-Remington et al. 1996). SE can adhere to and grow on polymer surfaces producing extracellular slime, a substance that obviously protects SE from host defense mechanisms and antimicrobials. We studied whether extracellular slime production of a *Staphylococcus epidermidis* strain has an effect on bacterial adherence to metallic surfaces in vitro.

### Material and methods

The influence of slime production on bacterial adherence to titanium discs in vitro was investigated by using *Staphylococcus epidermidis* RP 62 A, a slime-producing strain isolated from a polymer-associated infection (Christensen et al. 1982) and its isogenic, slime-negative mutant M7 (Schumacher-Perdreau et al. 1994). Strains were cultured (37 °C) in Trypticase Soy Broth/TSB (Becton Dickenson & Company/USA).

The primary bacterial suspension had an optical density of 0.45 arbitrary units at 580 nm. The colony-forming units of  $2 \times 10^7$  were determined by serial

plating dilution. The sterilized standardized pure titanium discs (1.5 cm diameter, 1 mm thickness/ Fr. Artos, Germany) were suspended on a sterile suture (3/0 thickness, VICRYL<sup>®</sup>) in the bacteria-containing TSB suspension. The discs did not touch the base or the side of the flask. 5 parallel probes were prepared.

The influence of incubation time and temperature for both strains was controlled by using three different incubation times and two different incubation temperatures. The incubation time of the discs in the bacterial suspension in the various experiments varied between 1.5, 6 and 24 hours. The incubation temperatures were 21° and 37 °C.

At the end of each incubation period, the 5 discs were taken out of the bacteria suspension and washed 4 times in 15 mL sterile saline solution to remove nonadherent bacteria.

Ultrasonification with a sonifier (Bronson 250/ USA) for 45 sec. per side with 80 watts was performed to release the bacteria adherent to the titanium discs. We then evaluated viable cells after plating serial dilutions on blood agar plates.

In the following, both strains are compared under resting and growing conditions. The data on the relation of adherent bacteria in comparison to the primary suspension are shown by means of the untransformed data. This way of presenting the data was chosen because the colony-forming units were not exactly the same for each condition.

Table 1. Relative colony counts for both strains at different incubation times (1.5 and 6 hours) and temperatures (21 °C and 37 °C), 8 times, 5 observations

Strain	Time	Temp.	Mean	SD	95% CI
M7	1.5	21	0.0097	0.0008	0.0087–0.011
M7	1.5	37	0.0073	0.0014	0.0056–0.0090
M7	6	21	0.0042	0.0002	0.0040–0.0045
M7	6	37	0.061	0.0040	0.056–0.066
RP 62 A	1.5	21	0.0051	0.0003	0.0048–0.0054
RP 62 A	1.5	37	0.0035	0.0004	0.0030–0.0040
RP 62 A	6	21	0.0036	0.0003	0.0032–0.0040
RP 62 A	6	37	0.033	0.0040	0.028–0.038

Table 2. 3-factor ANOVA model for the conditions: strains (RP 62 A; M7), times (1.5h; 6h) and temperature (21°; 37 °C) based on log<sub>e</sub> transformed data

Source	Pr > F
Strain	0.0001
Time	0.0001
Temp.	0.0001
Strain • Time	0.0001
Strain • Temp.	0.0002
Time • Temp.	0.0001
Strain • Time • Temp.	0.009

## Statistics

The colony counts were described by means, standard deviation (SD) and 95% confidence intervals (95% CI). A 3-factor ANOVA model was used to assess the changes in the colony counts caused by the incubation time (1.5 h; 6 h) and temperature (21 °C; 37 °C) on the strains (RP 62 A; M7). However, due to heteroscedasticity, the ANOVA was based on the log transformed data.

The prolonged incubation effect at 37 °C of RP 62 A versus M7 strain was compared with the Welsh test. P-values ≤ 0.05 were considered as significant differences.

## Results

### Resting conditions

*S. epidermidis* RP 62 A at 1.5 and 6 hours and 21 °C (resting conditions). The first experiment was performed to analyze the influence of time on bacterial adhesion under resting conditions (21°C). The duration varied from 1.5 to 6 hours. The experiment with short incubation time under resting conditions was performed to detect primary adhesion of the slime-producing strain RP 62 A. At 1.5 hours' incubation time, 0.005 (SD 0.0003) bacteria (RP 62 A) in relation to the primary suspension adhered to the discs. This figure fell to 0.004 (SD 0.0003) at 6 hours (Table 1).

*S. epidermidis* M 7 at 1.5 and 6 hours' incubation time and 21 °C. As the first series of experiments had proved that *S. epidermidis* RP 62 A was capable of primary adhesion (resting conditions), we repeated these experiments with the strain M7, the isogenic mutant of RP 62 A which lacked the ability to produce extracellular slime.

At 21 °C incubation temperature and 1.5 hours' incubation time, 0.0097 (SD 0.0008) bacteria in relation to the primary suspension of SE M7 were adherent to the titanium discs. A longer incubation time reduced

the number of relative adherent bacteria to 0.0042 (SD 0.0002) (Table 1).

### Growing conditions

*S. epidermidis* RP 62 A at 1.5, 6 and 24 hours' incubation time and 37 °C incubation temperature (bacterial proliferation). As the first experiment had shown a primary adhesion of the slime-producing strain under resting conditions, we used bacterial proliferation conditions (37 °C) in the second experiment.

Under these conditions, we found an accumulation of adherent bacteria that depended on the incubation time. There was a significant increase in adhesion of the slime-producing strain RP 62 A from 1.5 h, 6 hours' to 24 hours' (0.5 (SD 0.04)) incubation time under growth conditions (Table 1).

*S. epidermidis* M 7 at 1.5, 6 and 24 hours' incubation time and 37 °C incubation temperature. As the isogenic mutant M 7 showed primary adhesion under resting conditions, we repeated this experiment under growth conditions (37 °C) and prolonged incubation times (1.5, 6, 24 hours)

At 37 °C incubation temperature, we saw an increase in bacterial adherence from 1.5 h incubation time to 6 hours followed by a decline in adherent bacteria at 24 hours' incubation time (0.05 (SD 0.008)) (Table 1).

*The multifactorial interaction between the experimental conditions 1.5 and 6h incubation time (Tables 1 and 2).* The highest relative toxicity (highest number of colony-forming units in relation to the primary suspension) under these conditions for RP 62 A was after 6 hours' incubation at 37 °C. Both RP 62 A and the SE M7 strain showed the highest values after 6 hours' incubation at 37 °C. For both strains, the ability to adhere decreased from 1.5 hours to 6 hours at a temperature of 21 °C. It increased from 1.5 hours to 6 hours at the higher temperature of 37 °C (Table 1).

We observed a significantly (Welsh test,  $p = 0.0001$ ) lower mean relative bacterial adherence for SE M7 (0.05 (SD 0.008)) than for RP 62 A (0.5 (SD 0.04)) at 37 °C and 24 h' incubation time.

## Discussion

The frequency of pathological microorganisms in sepsis of totally replaced joints varies slightly (Inman et al. 1984, Fitzgerald and Jones 1985). Staphylococci including *S. aureus* and *S. epidermidis* comprise about half of the pathogens (Brause 1989). Charnley wrote in 1969 "It is a paradox that simple adherence to Listerian principles has abolished virulent postoperative infection, but infection by mildly pathogenic staphylococci appears to be increasingly common" (Charnley and Eftekhar 1969). Recently, Fitzgerald et al. (1994) proved the accuracy of this statement, showing that *S. epidermidis* was almost twice as common as *S. aureus* in 105 infected total joint arthroplasties, treated during 1989–1992.

While infections with *S. aureus* cause significant clinical signs, infections with *S. epidermidis* are often difficult to recognize. There is increasing evidence that prosthetic hip loosening may be caused by a low-grade infection with *S. epidermidis* (Rütt et al. 1990, Peters et al. 1991). *S. epidermidis* is known for its ability to adhere to various biomaterials, especially on polymer surfaces (Peters et al. 1982, Gristina et al. 1987, Perdreau-Remington et al. 1996). Barth et al. (1989) showed that there were lower numbers of a poor exopolysaccharide-producing strain of *S. epidermidis* (SP-2) on titanium-aluminum and polymethylmethacrylate than of a good exopolysaccharide-producing strain (SE-360). We believe that the use of an isogenic mutant strain of *S. epidermidis* (M7) lacking the ability of exopolysaccharide production, but being otherwise indistinguishable from the wild type, (RP 62 A) allows better interpretation of the results than the use of two different strains. Our experiments were done to show whether extracellular slime production affects the adherence of *S. epidermidis* to pure titanium, and to determine the influence of temperature and incubation time on these results in vitro.

Our findings demonstrate that *S. epidermidis* adheres to pure titanium surfaces. Both staphylococci strains primarily adhere to pure titanium. This ability is not dependent on slime production, as the slime-negative mutant *S. epidermidis* M 7 also adhered. This primary adherence is due to nonspecific hydrophobic interaction between the bacteria and the metallic surface. As the M7 strain cannot remain on the titanium surface, we found a reduction of adherent bacte-

ria at 6 hours under resting conditions.

Under the same conditions, the slime-producing strain RP 62 A could adhere firmly to the titanium discs. The decrease in bacterial adhesion after 6 hours for RP 62 A is not so high as for *S. epidermidis* M 7. RP 62 A is better able to stay on the metallic surface under resting conditions. This is due to an intercellular adhesion factor. Increase in incubation temperature at 1.5 hours' incubation time had no significant effect on bacterial adhesion of either of the strains. This indicates that primary adhesion is influenced not by temperature, but by nonspecific hydrophobic interaction between strain and biomaterial surface. Prolonged incubation time under growth conditions leads to higher colony counts of titanium-adherent bacteria of the slime-producing strain RP 62 A. We found a further increase in bacterial counts of the slime-producing strain (RP 62 A) on titanium discs from 6 to 24 hours under growth conditions.

Our findings illustrate the large effect of slime production on proliferation of the primary adherent bacteria on the titanium surface in vitro, as there was no accumulation of slime-negative bacteria. In vivo, the adherence is enforced by specific proteins like fibronectin, collagen, laminin and fibrinogen, which are important factors promoting adherence of gram-positive cocci to biomaterials (Christensen et al. 1989). The slime film protects *S. epidermidis* from antimicrobial drugs and may be an inhibitor of phagocytosis by polymorphonuclear neutrophils (PMN) (Johnson et al. 1986).

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