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Influence of batch or fed-batch growth on *Staphylococcus epidermidis* biofilm formation

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ABSTRACT

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Aims: To make a quantitative evaluation of the differences in biofilm formation by *Staphylococcus epidermidis* using batch and fed-batch growth systems and to correlate this with production of the major biofilm polysaccharide, poly-N-acetyl glucosamine (PNAG).

Methods and Results: Dry weight measurements of biofilms formed in batch and fed-batch conditions were compared with haemagglutination titres, which measure the amount of PNAG produced. Strains grown in batch systems developed less biofilm than when grown in fed-batch systems. A good correlation was found between the amount of biofilm formed in fed-batch systems and the haemagglutination titres.

Conclusions: Differences in biofilm formation and PNAG production by *S. epidermidis* are dependent on the availability of nutrients, with higher availability correlating with more biofilm and PNAG production.

Significance of and Impact of the Study: Comparisons of the formation of biofilms by *S. epidermidis* are dependent on choosing an appropriate biofilm growth system. Comparability or disparity of conclusions among different investigations will be strongly influenced by which mode *S. epidermidis* biofilms are formed.

Keywords: acrylic, haemagglutination, medical devices, nosocomial infections, PNAG, *Staphylococcus epidermidis*.

INTRODUCTION

Staphylococcus epidermidis and related coagulase-negative staphylococci (CoNS) are now well established as major nosocomial pathogens associated with infections of indwelling medical devices. Biofilm formation is one of the major virulence factors produced by these organisms (Voung and Otto 2002). In *S. epidermidis*, a polymer of N-acetyl glucosamine, initially defined biologically as the polysaccharide intercellular adhesin (PIA) and chemically as poly-*N*-acetyl-glucosamine (PNAG) has been identified as the molecule responsible for biofilm formation (Mack *et al.*, 1996; Heilmann *et al.* 1996; McKenney *et al.* 1998; Maira-Litrán *et al.* 2002). PNAG is also involved in the agglutination of erythrocytes, which is a common property of *S. epidermidis* strains (Mack *et al.* 1999; Joyce *et al.* 2003). This characteristic can be used to estimate the presence and level of PNAG produced by *S. epidermidis* strains using haemagglutination assays.

Several different methods have been developed for testing biofilm formation (Donlan and Costerton 2002) although most of them are based on a 1-day batch culture. However, in clinical situations biofilms develop over a long period of time, in conditions hardly similar to batch systems (Everaert *et al.* 1997).

The aim of this study was to evaluate the differences in the ability of six *S. epidermidis* strains to form a biofilm on acrylic, using distinct growth conditions. Biofilm formation following 1 or 3 days of interaction between bacteria and acrylic in batch mode were compared with biofilms formed in fed-batch mode.

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A correlation between the amount of biofilm formed in each situation with the haemagglutination titres was carried out to see if the differences in the amount of biofilm biomass formed correlated with production of PNAG.

MATERIALS AND METHODS

Bacteria strains

The *S. epidermidis* strains used in this work were isolated from the skin of healthy individuals: FJ6, JI6, LE7 and PE9. Two additional control strains were used: 9142, a strong biofilm producer that produces PNAG and an isogenic mutant, strain 9142-M10, which has a transposon inserted into the *ica* locus that encodes the proteins involved in PNAG synthesis and thus does not produce a PNAG-based biofilm. These control strains were provided by D. Mack (Hamburg, Germany).

Media and Growth conditions

TSB and TSA (Merck, Darmstadt, Germany) were prepared according to the manufacturer instruction. Physiological saline was prepared adding 0·9% of NaCl (Merck) to distilled water.

All strains were incubated in 15 ml of TSB inoculated from TSA plates not older than 2 days, for 24 (±2) h at 37°C with agitation of 130 rev min^{-1}, in an orbital shaker (SI50; Stuart Scientific, Redhill, UK). Then, 50 μl were transferred to 30 ml of fresh TSB, and allowed to grow for 18 (±2) h, at 37°C with agitation of 130 rev min^{-1} before cells were harvested by centrifugation (Sigma 4K10, B. Braun, Germany) for 5 min at 10 500 *g* and 4°C. Cells were then resuspended in physiological saline at a density of *ca* 1×10^9 cells ml^{-1}.

Surface preparation

Acrylic (Repsol, Brønderslen, Denmark) was cut into 2 cm × 2 cm surfaces and then immersed in a 0·2% commercial detergent solution (Sonazol Pril, Alverca, Portugal) overnight, after which the surfaces were transferred to a new 0·2% commercial detergent solution, prepared in warm water, which were well agitated for 5 min. The detergent was removed by thoroughly rinsing with distilled water. Finally, each individual surface was rinsed thoroughly with ultrapure water and sterilized by immersion in a flask filled with distilled water and autoclaved for 15 min at 121°C.

Biofilms formed in batch mode

Sterilized acrylic surfaces were inserted in each well of a six-well tissue culture plates (Sarstedt, Newton, NC, USA) containing 5 ml of TSB supplemented with 0·25% of glucose (Merck). Then 20 μl of a 0·9% NaCl solution containing 1×10^9 cells ml^{-1} was added and growth was allowed to occur during 24 or 72 h, at 37°C, in a shaker at 120 rev min^{-1}. Negative controls were obtained by incubating the surfaces in TSB supplemented with 0·25% glucose without adding any bacterial cells. All experiments were performed in quadruplicate with three repeats.

Biofilms formed in fed-batch mode

Biofilms were formed on sterilized acrylic surfaces as described above, except that at every 12 h the TSB medium containing suspended bacterial cells was removed and an equal volume of fresh TSB with 0·25% of glucose was added. Negative controls were obtained by incubating the surfaces in TSB with 0·25% glucose without adding any bacterial cells. All experiments were performed in quadruplicate with three repeats.

Biofilm dry weight determination

Biofilm dry weight determinations were performed as previously described (An and Friedman 1997) with some modifications. Briefly, the colonized surfaces were removed from the plates and placed at 80°C overnight. Then the weight of the surface was determined on a digital scale (BL120S; Sartorius, Goettingen, Germany). Surfaces were placed again at 80°C for two more hours, and weighed again, to check the stability of the dry weight. Then, the biofilm was mechanically removed from the surface, and the surfaces were thoroughly cleaned with 0·2% commercial detergent solution (Sonazol Pril). Cleaned surfaces were kept overnight at 80°C, prior to a third weight determination. The difference in the weight of the surface with and without the biomass attached is the biofilm dry weight.

Haemagglutination assays

The haemagglutination assays were performed as previously described (Rupp and Archer 1992) with some modifications. Briefly, *S. epidermidis* biofilm was formed in 96-well (U-shaped) microtitre plates for 24 h, at 150 rev min^{-1} and 37°C, in TSB supplemented with 0·25% glucose. Planktonic cells were then removed and the biofilm was scraped off from the surface and resuspended in saline, followed by sonication (20 W, 20 s) for disrupting the cell aggregates. The cell density was then adjusted to a concentration of *ca* 3×10^9 cells ml^{-1}. Five two-fold dilutions of each cell suspension were made (100 μl) in 96 well (U-shaped) microtitre plates. Then 100 μl of a 1% horse erythrocyte solution was added to each well. To ensure thorough mixing of the bacteria and erythrocytes, the total

volume of each well was pipetted in and out with a micropipette. Incubation was at room temperature for 2 h, and haemagglutination titres were evaluated macroscopically. All experiments were performed in duplicate with three repeats.

Statistical analysis

All the biofilm assays were compared by the one-way analysis of variance by applying the Levene's test of homogeneity of variances and the Tukey multiple comparisons test, and also paired *t*-test, using SPSS (Statistical Package for the Social Sciences). All tests were performed with a confidence level of 95%.

RESULTS

Biofilm formation

Table 1 presents the amount of biofilm formed in the three conditions assayed in this study. The PNAG-negative control strain 9142-M10 hardly formed any biofilm, whereas the parental PNAG-positive control strain, 9142, formed biofilms in all conditions assayed. In batch mode, for all four isolates strains, there was a slight increase in the dry weight of the biofilm after 72 h compared with 24 h ($P < 0{\cdot}05$, paired *t*-test). With this method, no significant differences ($P > 0{\cdot}05$, ANOVA and Tukey's multiple comparison test) were found in the abilities of the four strains to form biofilms. However, when the fed-batch approach was used, in addition to producing more biofilm, significant differences ($P < 0{\cdot}05$, ANOVA and Tukey's multiple comparison test) were found among the isolates, with both strains JI6 and PE9 producing more biofilm than strains FJ6 and LE7, as did the positive control strain 9142.

Table 1 Biofilm formation on acrylic under the different conditions used, in μg biofilm mass mm^{-2} of acrylic

Strain	24 h batch	72 h batch	72 h fed-batch
9142	1·5 (±0·4)*	4·9 (±1·4)*	16·8 (±4·5)†
9142-M10	0·2 (±0·1)	0·5 (±0·2)	1·2 (±0·5)
FJ6	2·6 (±0·4)*	4·2 (±1·2)*	5·6 (±2·2)
JI6	2·1 (±0·4)*	3·6 (±1·4)*	10·2 (±2·3)†
LE7	1·9 (±0·4)*	3·8 (±1·3)*	4·2 (±2·3)
PE9	2·5 (±0·4)*	5·6 (±1·0)*	12·4 (±4·8)†

*The 24 h batch is significantly different from 72 h batch ($P < 0{\cdot}05$, paired *t*-test).
†Significantly higher biofilm formation compared with other strains grown under the same conditions ($P < 0{\cdot}05$, ANOVA and Tukey's multiple comparison test).

Table 2 Haemagglutination titres of the *Staphylococcus epidermidis* biofilms

Strain	Haemagglutination titres
9142	1:8
9142-M10	No haemagglutination
FJ6	1:1
JI6	1:8
LE7	1:1
PE9	1:8

Haemagglutination titre

Table 2 presents the haemagglutination titres of the four *S. epidermidis* isolates and also the two control strains. The biofilm positive control strain presented a haemagglutination titre of 1 : 8, indicating it took nearly $3{\cdot}7 \times 10^8$ cells ml^{-1} to cause agglutination of horse erythrocytes. However, the biofilm negative control strain was not able to agglutinate horse erythrocytes.

All four *S. epidermidis* isolates were able to cause haemagglutination. PE9 was most efficient at causing haemagglutination, as only $3{\cdot}7 \times 10^8$ cells ml^{-1} were needed to agglutinate horse erythrocytes. Conversely, strain LE7 and JI6 were the least efficient strains in causing haemagglutination since at least $3{\cdot}0 \times 10^9$ cells ml^{-1} were needed to agglutinate horse erythrocytes.

A linear regression analysis was made between the amount of biofilm formed under different growth conditions and the haemagglutination titres. The correlation coefficient (R) between haemagglutination titre and biofilm mass for biofilms formed in batch mode and grown for 24 or 72 h were 0·28 and 0·78, respectively, whereas the correlation between haemagglutination and biofilm mass for fed-batch biofilms was 0·94.

DISCUSSION

Some investigations into the ability of *S. epidermidis* strains to form biofilms on inert surfaces and correlations with production of surface factors have been published (Cramton *et al.* 1999; Hume *et al.* 2004). However, there is not always agreement in the conclusions regarding biofilm formation, likely because of use of different systems by different investigators to form biofilms (Neu *et al.* 1994; Pitts *et al.* 2003; Hume *et al.* 2004). Thus, in order to make meaningful comparisons among different studies, it is important to determine which methodological differences can account for apparently disparate results.

In this study, the amount of biofilm formed on acrylic using distinct growth conditions was compared and these findings were correlated with the haemagglutination titre, a

measure of the production of PNAG (Mack *et al.* 1999). Since biofilms formed *in vivo* in an infected host develop over a long period of time, and biofilm properties change with age (Neu *et al.* 1994), it was hypothesized that changes in availability of nutrients over time could influence the amount of a *S. epidermidis* biofilm. When biofilms were formed under batch mode, the amount of biofilm slightly increased with the biofilm age. However, no statistically significant differences were found between the amount of biofilm formed among the isolates strains. Also, the correlation between biofilm biomass and haemagglutination titre was low.

Conversely, in the fed-batch systems, there was a higher amount of biofilm formed compared with the batch mode and clear differences in the amount of biofilm produced among strains were obtained ($P < 0{\cdot}05$, ANOVA and Tukey's multiple comparison test). Thus, the fed-batch system revealed differences in biofilm formation that were not apparent with the standard batch system. A strong linear relation was found between fed-batch biofilm formation and haemagglutination titres, indicating in this system PNAG may have made more of a contribution to formation of the biofilm compared with its contribution in the standard batch system.

Overall, in comparing biofilm biomass production by *S. epidermidis* in a standard batch system to a fed-batch system, produced by adding fresh medium, biofilm formation occurred to a greater extent in the latter system and correlated with the amount of PNAG produced. These findings indicate that attention must be taken in choosing the growth method used to measure biofilm production by *S. epidermidis* in order to make meaningful comparisons among different investigations. Biofilm formation by medically relevant *S. epidermidis* is used in many studies, including those looking for molecular and cellular correlates of biofilm formation (Heilmann *et al.* 1996; Cramton *et al.* 1999; Galbart *et al.* 2000), in studies concerning prevention of biofilm formation by antimicrobial agents (Rupp and Hamer 1998) or to evaluate some host immune factors (Mohamed *et al.* 1999). As many of these studies attempt to evaluate the role of PNAG in the pathogenesis of infection and in biofilm formation properties, the use of static-batch systems may not be suitable. Alternately, a fed-batch system for making comparisons among strains in their abilities to form biofilms may be more relevant to studies evaluating a role of PNAG in this process. Most importantly, studies reporting differences in a role of PNAG in biofilm formation, occurrence and role in pathogenesis may be strongly influenced by several factors such as those related to growth medium and availability of nutrients. Careful attention and standardization of the biofilm formation mode will be highly useful for making proper comparisons among different investigations evaluating a role for PNAG and biofilm in the pathogenesis of *S. epidermidis* infection.

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REFERENCES

An, Y.H. and Friedman, R.J. (1997) Laboratory methods for studies of bacterial adhesion. *Journal of Microbiological Methods* **30**, 141–152.

Cramton, S.E, Gerke, C., Schnell, N.F., Nichols, W.W. and Gotz, F. (1999) The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infection and Immunity* **67**, 5427–5433.

Donlan, R.M. and Costerton, J.W. (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews* **15**, 167–193.

Everaert, E.P., Mahieu, H.F., Chung, R.P., Verkerke, G.J., van der Mei, H.C. and Bussher, H.J. (1997) A new method for in vivo evaluation of biofilms on surface-modified silicone rubber in a parallel plate flow chamber in the absence and presence of nutrient broth. *Microbiology* **143**, 1569–1574.

Galbart, J., Allignet, J., Tung, H, Rydèn, C. and Solh, N.E. (2000) Screening for *Staphylococcus epidermidis* markers discriminating between skin-flora strains and those responsible for infections of joint prostheses. *Journal of Infectious Diseases* **182**, 351–355.

Heilmann, C., Gerke, C., Perdreau-Remington, F. and Gotz, F. (1996) Characterization of Tn917 insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. *Infection and Immunity* **64**, 277–282.

Hume, E., Baveja, J., Muirf, B., Schubert, T., Kumar, N. Kjelleberg, S., Griesser, H., Thissen, H. *et al.* (2004) The control of *Staphylococcus epidermidis* biofilm formation and in vivo infection rates by covalently bound furanones. *Biomaterials* **25**, 5023–5030.

Joyce, J., Abeygunawardana, C., Xu, Q., Cook, J., Hapler, R., Prysiecki, C., Grim, K., Roper, K *et al.* (2003) Isolation, structural characterization, and immunological evaluation of a high-molecular weight exopolysaccharide from *Staphylococcus aureus*. *Carbohydrate Research* **338**, 903–922.

Mack, D., Haeder, M., Siemssen, N. and Laufs, R. (1996) Association of biofilm production of coagulase-negative staphylococci with expression of a specific polysaccharide intercellular adhesin. *Journal of Infectious Diseases* **174**, 881–884.

Mack, D., Riedwald, J., Rohde, H., Magnus, T., Feucht, H., Elsner, H., Laufs, R. and Rupp, M. (1999) Essential functional role of the polysaccharide intercellular adhesin of *Staphylococcus epidermidis* in hemagglutination. *Infection and Immunity* **67**, 1004–1008.

Maira-Litrán, T., Kropec, A., Abeygunawaerdana, C., Joyce, J., Mark, G., Goldman, D. and Pier, G. (2002) Immunochemical properties of the staphylococcal poly-N-acetylglucosamine surface polysaccharide. *Infection and Immunity* **70**, 4433–4440.

McKenney, D., Hubner, J., Muller, E., Wang, Y., Goldmann, D.A. and Pier, G.B. (1998) The ica locus of *Staphylococcus epidermidis*

encodes production of the capsular polysaccharide/adhesin. *Infection and Immunity* **66**, 4711–4720.

Mohamed, N., Teeters, M., Patti, J., Hook, M. and Ross, J. (1999) Inhibition of *Staphylococcus aureus* adherence to collagen under dynamic conditions. *Infection and Immunity* **67**, 589–594.

Neu, T.R., De Boer, C.E., Ververke, G.D., Schutte, H.K., Rakhorst, G., van der Mei, H.C. and Busscher, H.J. (1994) Film development in time on a silicone rubber voice prosthesis – a case study. *Microbiology and Ecology in Health Diseases* **7**, 27–33.

Pitts, B., Hamilton, M., Zelver, N. and Stewart, P. (2003) A microtiter-plate screening method for biofilm disinfection and removal. *Journal of Microbiological Methods* **54**, 269–276.

Rupp, M. and Archer, G. (1992) Hemagglutination and adherence to plastics by *Staphylococcus epidermidis*. *Infection and Immunity* **60**, 4322–4327.

Rupp, M. and Hamer, K.E. (1998) Effect of subinhibitory concentrations of vancomycin, cefazolin, oflaxacin, l-oflaxacin and d-oflaxacin on adherence to intravascular catheters and biofilm formation by *Staphylococcus epidermidis*. *Journal of antimicrobial chemotherapy* **41**, 155–161.

Voung, C. and Otto, M. (2002) *Staphylococcus epidermidis* infections. *Microbes and Infection* **4**, 481–489.