

THE DYNAMICS OF *STAPHYLOCOCCUS EPIDERMIS* BIOFILM FORMATION IN RELATION TO NUTRITION, TEMPERATURE, AND TIME

HOLÁ V., RŮŽIČKA F., VOTAVA M.

Department of Microbiology, Faculty of Medicine, Masaryk University, Brno,

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Abstract

The ability to form biofilm is one of the most important virulence factors occurring in microbes. The biofilm-forming bacteria are difficult to eradicate with antibiotics and often cause chronic infections. The aim of this study was to correlate the dynamics of biofilm formation with nutrition and temperature conditions during the cultivation in biofilm-positive *Staphylococcus epidermidis* isolates. The cultivation was performed on standard microtitre plates and the wells were examined for the production of biofilm every 1 hr up to 48 hrs. All the tested strains showed better growth of the biofilm at a temperature of 37 °C in a nutrient-rich environment. The first signs of bacterial adhesion were visible after 2-4 hours of cultivation, the first homogenous but a very thin layer was visible after 5 hours; after 7 hours the biofilm layer was circa three times thicker. After 10 hours the biofilm layer seemed to be mature - the changes in thickness were not so evident after this time. After a cultivation longer than 34-42 hours, parts of the biofilm layer started to detach, and consequently the biofilm became non-homogeneous. For biofilm studies, a biofilm cultivation for 12-16 hours should be sufficient.

Key words

Biofilm formation, Staphylococci, Nutrition, Glucose, Dynamics

INTRODUCTION

The ability to form biofilm, a slimy layer with embedded microcolonies, is one of the most important and one of the most widespread virulence factors occurring in microbes. This ability can be found in bacteria living in the outer environment as well as in pathogens and potential pathogens of humans. The biofilm-forming ability helps bacteria to resist the conditions of the surrounding environment. Biofilms grow easily on surfaces of artificial materials used for catheters and prosthetic devices (1), and it is estimated that biofilms are associated with about 65 % of nosocomial infections (2). These infections are usually chronic and difficult to treat. The higher incidence of biofilm-associated infections is associated with the frequent use of artificial implants and medical devices nowadays, and bacteria most commonly isolated from these infections are those of the *Staphylococcus* genus.

The knowledge of biofilm formation dynamics is fundamental for the understanding of processes running in the biofilm layer. There are many works that discuss some features of biofilm-positive bacteria, but there is no consistency in the conditions which are feasible for biofilm formation among authors (3-6). The only agreement is in the culture temperature, 37°C seems to be appropriate. Other conditions, e.g. presence of nutrition and time of cultivation, vary in many publications. In our study we paid attention to those culture conditions that differ in most authors. The aim of this study was to determine the differences in biofilm formation in different conditions and to determine the minimum time and conditions necessary for the development of a homogenous and mature biofilm layer.

MATERIALS AND METHODS

Twenty-three biofilm-positive *Staphylococcus epidermidis* strains were used in this study. One reference strain (*S. epidermidis* No. CCM 7221, Czech Collection of Microorganisms, Brno, CZ) was used as a positive control. The 22 clinical strains were isolated from blood cultures and indwelling medical devices of patients with clinical and laboratory markers of sepsis (bacteraemia). The isolates were identified at the species level using a STAPHYtest kit (Lachema, Brno, CZ). The identification was verified by proof of *S. epidermidis*-specific DNA by the PCR method.

The ability to form biofilm was assessed by both phenotypic and genotypic methods. The presence of the intact *ica* ADB gene cluster, which is responsible for biofilm formation in staphylococci, was proved by the amplification of DNA by PCR with primers designed by *Frebourg et al.* (7) and with a final length of the amplification product of 546 bp. To avoid false-negative results caused by inhibition of the reaction, amplification of the specific sequence of *S. epidermidis* with primers SE705-1 and SE705-2 (8) of a length of the amplification product of 124 bp was used as the control of the reaction. The reaction was performed in a Progene thermocycler (Techne, Cambridge, UK), using Taq DNA polymerase (Invitrogene, Carlsbad, USA) and dNTP mix (Invitrogene, Carlsbad, USA).

Phenotypically, biofilm formation was assessed by a modified Christensen test tube method (9), where the Brain Heart Infusion (Himedia, Mumbai, India) was used instead of the Tryptic Soya Broth medium. All strains were positive in both of the above-mentioned methods.

The biofilm was grown on 96-well flat-bottomed polystyrene tissue culture microtitre plates (GAMA Group, Trhové Sviny, CZ). A fresh overnight culture of *S. epidermidis* from the blood agar was suspended and vortexed in phosphate buffered saline to an optical density of 0.5 of the McFarland scale. Twenty microlitres of this suspension was inoculated into the wells of a microtitre plate with 180 µl of the tested medium. The medium - Brain Heart Infusion (BHI; Himedia, Mumbai, India) - was supplemented with different concentrations of glucose (0 %, 4 %, and 9 %) to obtain three media differing in nutrition richness. The negative control wells contained only the tested media. All the tested strains were cultured at 37 °C, and at 25 °C in aerobic conditions. In order to obtain average values of the optical density, each strain was cultivated simultaneously in 4 wells. Every hour the four wells of the microtitre plate were emptied, washed three times with phosphate buffered saline (pH 7.4), and fixed by drying (10). The study was designed to cover 48 hours of cultivation. After the cultivation and fixation the adhered biofilm layer was stained for 20 minutes with 160 µl of 0.7 % crystal violet and then the rest of the dye was rinsed off under running tap water. The plates were air-dried and for the spectrophotometric assessment the bound dye was re-dissolved with 160 µl of 75 % ethanol per well. The optical density of each well was measured at 620 nm using an Anthos Labtec Instruments 2001 reader (Salzburg, Austria).

The data obtained were processed with the Multiple Analysis of Variance (MANOVA) and values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

All the tested strains showed a better and richer growth of the biofilm layer at a temperature of 37 °C in the nutrient-rich environment (*Fig. 1*). The model can be simply described as follows: the first signs of bacterial adhesion were visible with the naked eye after 2–4 hours of cultivation, the first homogenous but a very thin layer was visible after 5 hours; after 7 hours the biofilm layer was circa three times thicker. After 12–14 hours the biofilm layer seemed to be mature – the changes in the thickness were not so evident after this time. After a cultivation longer than 34–42 hours parts of the biofilm layer started to detach, so the biofilm layer became non-homogeneous.

At a temperature of 25 °C the mass of the formed biofilm was approximately four times smaller. No differences in biofilm formation in different supplementations of glucose were evident (*Fig. 2*) and significant in the statistical analysis (Multifactorial ANOVA, programme R: A Language and Environment for Statistical Computing 2.1.1; $p=0.32$). The formation of the biofilm layer was slow and the maximum optical density (OD) in BHI supplemented with 9 % of glucose reached only circa 0.600. The growth curve of biofilm formation was nearly linear. The supplementation of BHI with glucose does not seem to affect biofilm formation ($p = 0.05$) in these conditions.

The results of our experiments show that there are great differences in the mass of the biofilm formed with regard to the different culture glucose supplementation. We can say that the temperature of 25°C is insofar low as the bacteria are only surviving and their mass is growing very slowly. The mass of the formed biofilm in these conditions (*Fig. 2*) was not increased even by glucose supplementation. At a temperature of 37 °C the differences among the differently supplemented media are more obvious and statistically significant. Here we can observe several phases of biofilm layer formation. The exponential phase begins just after the inoculation and lasts for circa 12 hours. After this time we can see a slowing down of the bacterial growth. At this phase we suppose that the equilibrium of a mature biofilm was reached. There is a dynamic balance in the biofilm layer and the mass of the biofilm is not growing, or is not growing so rapidly. Some authors suppose that at this phase of biofilm formation the upper layer of the biofilm starts to generate planktonic cells, which may colonise other solid surfaces (*11*).

Once the bacteria have saturated the environment, the biofilm starts to deteriorate. This may be caused by the formation of resting cells (*12*) or by the starvation caused by deficiency of nutrients, decreased perfusion in the biofilm layer, low pH of the environment, a decrease of the partial pressure of oxygen, or by accumulation of toxic by-products of bacterial metabolism. By these changes we can explain the detachment of large parts of the biofilm layer from the solid surface, observable after 34 hours of cultivation. The starvation and withering of the cells seem to be strongest in the lower biofilm layers, where the bacteria are attached to a solid sur

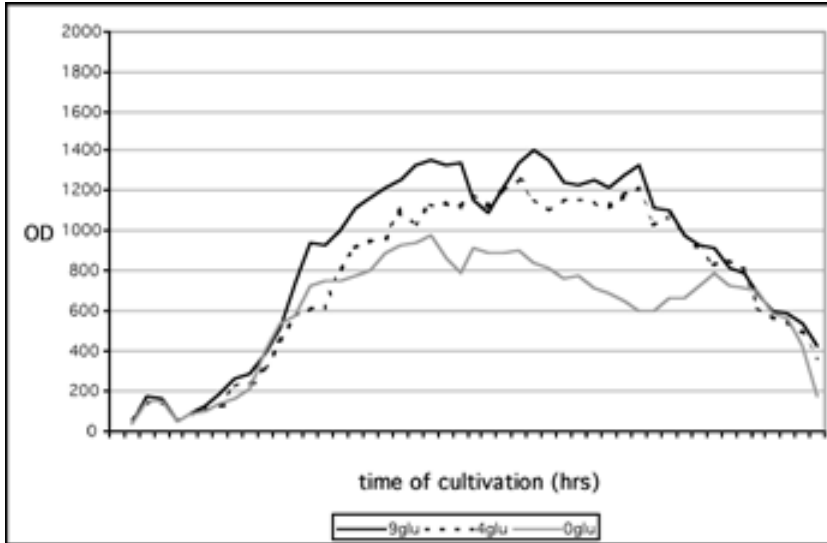


Fig. 1

The dynamics of biofilm formation at 37 °C

Key: 9glu - BHI supplemented with 9% of glucose; 4glu - BHI supplemented with 4% of glucose; 0glu - standard BHI; axis x - time of cultivation (hours); axis y - optical density (OD)

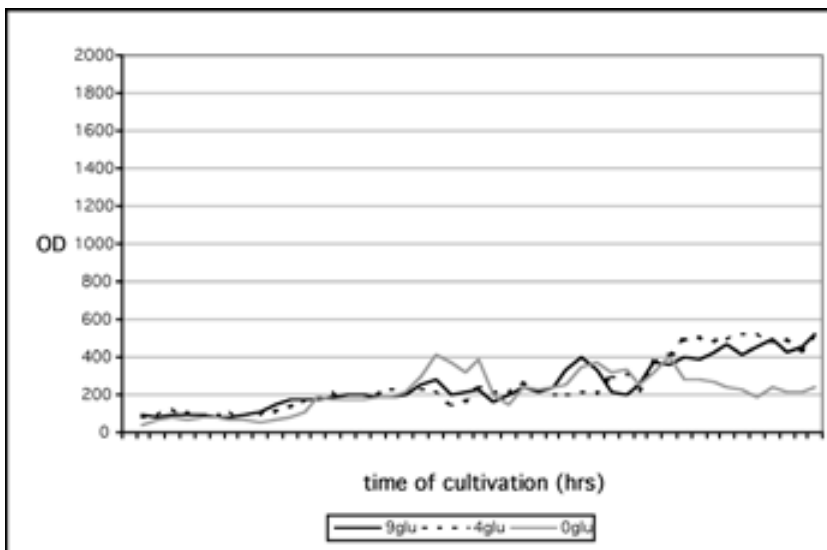


Fig. 2

The dynamics of biofilm formation at 25 °C

Key: 9glu - BHI supplemented with 9% of glucose; 4glu - BHI supplemented with 4% of glucose; 0glu - standard BHI; axis x - time of cultivation (hours); axis y - optical density (OD)

face and are dependent on the nutrition and oxygen coming through the whole layer of the biofilm. In these layers the bacterial population starts to decrease and only extracellular polysaccharides and dead bacterial cells remain. The division of new cells is limited as well, because of starvation. The biofilm layer may then easily fall apart. We observed the disruption of large parts of the biofilm layer in all three nutrition forms of BHI, but the strongest effect was observable in a medium supplemented with 9 % of glucose.

The results of our experiment can be concluded as follows: The growth optimum in staphylococci is not affected by biofilm formation, and the ability to form biofilm did not help to resist the discomfort of different culture temperatures. The optimal temperature for biofilm formation is 37 °C. At 25 °C the amount of the grown biofilm is lower during the same time period. The biofilm is mature after 12 hours, and after a cultivation longer than 34 hours it starts to disrupt. For the biofilm studies, e.g. antibiotic testing, a biofilm cultivation for 12–16 hours should be sufficient.

A c k n o w l e d g e m e n t

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Holá V., Růžička F., Votava M.

DYNAMIKA TVORBY BIOFILMU U *STAPHYLOCOCCUS EPIDERMIDIS* V ZÁVISLOSTI NA MNOŽSTVÍ ŽIVIN, TEPLOTĚ A NA ČASE

S o u h r n

Schopnost tvorby biofilmu je u mikrobů jedním z nejdůležitějších faktorů virulence. Infekce způsobené biofilmopozitivními bakteriemi se obtížně léčí antibiotiky a často přecházejí do chronicity. Cílem této studie bylo posoudit závislost tvorby biofilmu na množství živin a teplotě kultivace u biofilmopozitivních kmenů *Staphylococcus epidermidis*. Dynamika byla sledována v průběhu 48 hodin, a to každou celou hodinu. U všech testovaných kmenů byla tvorba biofilmu nejlepší při 37 °C v nutričně bohatších médiích. První znaky bakteriální adheze byly pozorovatelné po 2 – 4 hodinách kultivace, první homogenní, i když velmi slabá biofilmová vrstva byla pozorovatelná po pěti hodinách, po sedmi hodinách byla vrstva biofilmu cca třikrát silnější. Po 10 – 12 hodinách kultivace se biofilmová vrstva zdála již stabilní a změny v tloušťce biofilmové vrstvy po této době nebyly již tak výrazné. Po 34 – 42 hodinách kultivace se začaly části biofilmové vrstvy odlupovat a biofilmová vrstva se stala nehomogenní. Pro studium vlastností biofilmu se jako dostatečná jeví doba kultivace 12 – 16 hodin.

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