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REVIEW

Extracellular DNA in biofilms

Lucio Montanaro[1,2], Alessandro Poggi[1,3], Livia Visai[4,5,6], Stefano Ravaioli[1,2], Davide Campoccia[1], Pietro Speziale[4], Carla Renata Arciola[1,2]

[1]Research Unit on Implant Infections, Rizzoli Orthopaedic Institute, Bologna - Italy
[2]Department of Experimental Pathology, University of Bologna, Bologna - Italy
[3]Medicine and Surgery School, University of Modena and Reggio Emilia, Modena - Italy
[4]Department of Biochemistry, University of Pavia, Pavia - Italy
[5]Center for Tissue Engineering (C.I.T.), University of Pavia, and Salvatore Maugeri Foundation IRCCS, Pavia - Italy
[6]International Center For Studies and Research in Biomedicine (I.C.B.) - Luxembourg

ABSTRACT

Extracellular DNA (eDNA) is an important biofilm component that was recently discovered. Its presence has been initially observed in biofilms of Pseudomonas aeruginosa*,* Streptococcus intermedius, Streptococcus mutans*, then* Enterococcus faecalis *and staphylococci. Autolysis is the common mechanism by which eDNA is released. In P. aeruginosa eDNA is generated by lysis of a bacterial subpopulation, under control of quorum sensing system. In* E. faecalis *autolysis proceeds in a fratricide mode, resulting from a process similar to necrosis of eukaryotic cells. In* Staphylococcus aureus *autolysis originates by an altruistic suicide, i.e., a programmed cell death similar to apoptosis of eukaryotic cells. In* S. aureus *autolysis is mediated by murein hydrolase, while in* S. epidermidis *by the autolysin protein AtlE. In* P. aeruginosa *eDNA is located primarily in the stalks of mushroom-shaped multicellular structures. In* S. aureus *the crucial role of eDNA in stabilizing biofilm is highlighted by the disgregating effect of DNase I. eDNA represents an important mechanism for horizontal gene transfer in bacteria. eDNA and other microbial structural motifs are recognized by the innate immune system via the TLR family of pattern recognition receptors (PRRs).*

KEY WORDS: *Extracellular DNA (eDNA), Bacterial biofilm,* Pseudomonas aeruginosa, Enterococcus faecalis, Staphylococcus aureus, S. epidermidis

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INTRODUCTION

Bacteria live in well-developed biofilms on the surfaces of infected indwelling medical implants and in bone affected by chronic osteomyelitis. Biofilm is a microbial derived sessile community characterized by cells that are firmly attached to a substratum, embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (1, 2).

The extracellular composition of biofilm is complex. Although initially the extracellular polymeric substance was considered to consist of extracellular polysaccharides, recently the bacterial extracellular matrix (ECM) has been found to be more complex, including lipopolysaccharides, glycolipids, lipids, proteins, and nucleic acids. The chemical composition of a biofilm depends on microbial genetics and on the environment in which bacteria grow and in which the biofilm matrix develops. Therefore the environmental conditions are what ultimately determine the properties of the biofilms.

For a long time studies have been focused on the polysaccharide intercellular adhesin (PIA) of *S. epidermidis* and *S. aureus* biofilms, encoded by the *icaADBC* locus, considered the major cell-to-cell connecting substance. In addition to the PIA polymer of *Staphylococcus aureus* and *Staphylococcus epidermidis*, in Gram-negative bacteria colanic acid *(E. coli)*, alginate, glucose- and mannose-rich matrix components *(Pseudomonas aeruginosa)*, cellulose and β-1,6-GlcNac polymer *(E. coli)*, have been reported to participate to the extracellular polymeric substance of biofilms (2).

But now attention is focused on another biofilm matrix component, extracellular DNA (eDNA), which has been shown to be important for biofilm formation. Initially, the presence of eDNA in biofilms of *P. aeruginosa*, *Streptococcus intermedius* and *Streptococcus mutans* were described (3). Starting from the observations of Arciola et al on strong biofilm production by epidemic clones of *Enterococcus faecalis* (4), Thomas et al have described the relationship between DNA release, role of proteases and biofilm production in *E. faecalis* (5, 6). After having given evidence that the mechanisms by which eDNA originates is autolysis, they advanced the concept of two modes of autolysis: an altruistic suicide and a fratricide killing of different sub-populations of bacterial cells. In the case of altruistic suicide (*S. aureus*), cells may be divided into altruists and survivors. Altruists commit suicide by programmed cell death (a process similar to apoptosis in eukaryotic cells) for the common sake of the larger community. In the case of fratricide (*E. faecalis*, *B. subtilis* and *S. pneumoniae*), cells differentiate into attackers and targets. Attackers release killing factors that kill targets (a process similar to necrosis in eukaryotic cells). The attackers themselves are protected from self-destruction by specific immunity proteins they express.

The production mode of eDNA by *P. aeruginosa* has been well characterized (7). In biofilm-producing *P. aeruginosa*, the extracellular DNA is generated by the lysis of a bacterial subpopulation. Cell lysis is controlled by *quorum sensing* systems, based on acyl homoserine lactone (AHL) and on *Pseudomonas* quinolone signaling (PQS). An abundant generation of eDNA was observed in late-log phase cultures, as expected for a *quorum sensing*-regulated process. *Quorum sensing* mutants did not release a large amount of DNA in the late-log phase, while accumulation of extracellular DNA in the late-log phase was restored upon supplementation with signal molecules. Moreover, accumulation of extracellular DNA was inhibited in the wild-type *Pseudomonas* culture when furanone (a specific *quorum sensing* inhibitor) was added. The presence of eDNA in *Pseudomonas* biofilms seems to have a stabilizing role, since biofilms of mutants poor in eDNA were more susceptible to the action of a disgregating agent. The authors conclude that the extracellular DNA in *P. aeruginosa* biofilms may be generated by at least two different pathways. A pathway not linked to *quorum sensing* is responsible for a basal level production of eDNA. Instead, the abundant eDNA released in the late-log phase is generated by *quorum sensing*-dependent mechanisms.

In staphylococci, the production of extracellular DNA is different and, although bacterial autolysis is implicated both in *S. epidermidis* and in *S. aureus*, different mechanisms are involved in the two species.

Qin et al have provided evidence that extracellular DNA is important for the initial phase of biofilm development by *S. epidermidis* (8). They found that extracellular DNA is a major component required for initial bacterial attachment to surfaces, as well as for the subsequent early phase of biofilm development. The similarity between extracellular DNA and chromosomal DNA was demonstrated by comparative PCR amplification of different genes (*yycF*, *trpS*, *srrA*, *sigB* and i*saA)*, which are located in different regions of the *S. epidermidis* chromosome. This observation strengthens the hypothesis that eDNA originates from lysis of a small subpopulation of the *S. epidermidis* bacteria. Bacterial autolysin AtlE was investigated for its role in biofilm formation and eDNA release. The authors constructed a defective *atlE* mutant strain and compared the amounts of eDNA in cultures of the Δ*atlE* and in parental strains. In cultures of Δ*atlE* strain the amount of extracellular DNA was dramatically decreased. Also biofilm formation was impaired in the Δ*atlE* strain. By florescence staining and CLSM analysis, the eDNA was found to surround the wild-type adhering cells in the initial phase of biofilm development, whereas eDNA was not detected on the surface of the few adhering cells of the Δ*atlE* strain. The elegant research of Qin et al provided evidence that (i) extracellular DNA is a major component contributing to initial attachment of *S. epidermidis* to glass or plastic surfaces; (ii) DNase I treatment prevented *S. epidermidis* attachment and biofilm formation; (iii) DNA release from *S. epidermidis* appears to be mainly mediated by the autolysin protein AtlE, since inactivation of *atlE* drastically reduced DNA release. The authors suggest that AtlE,

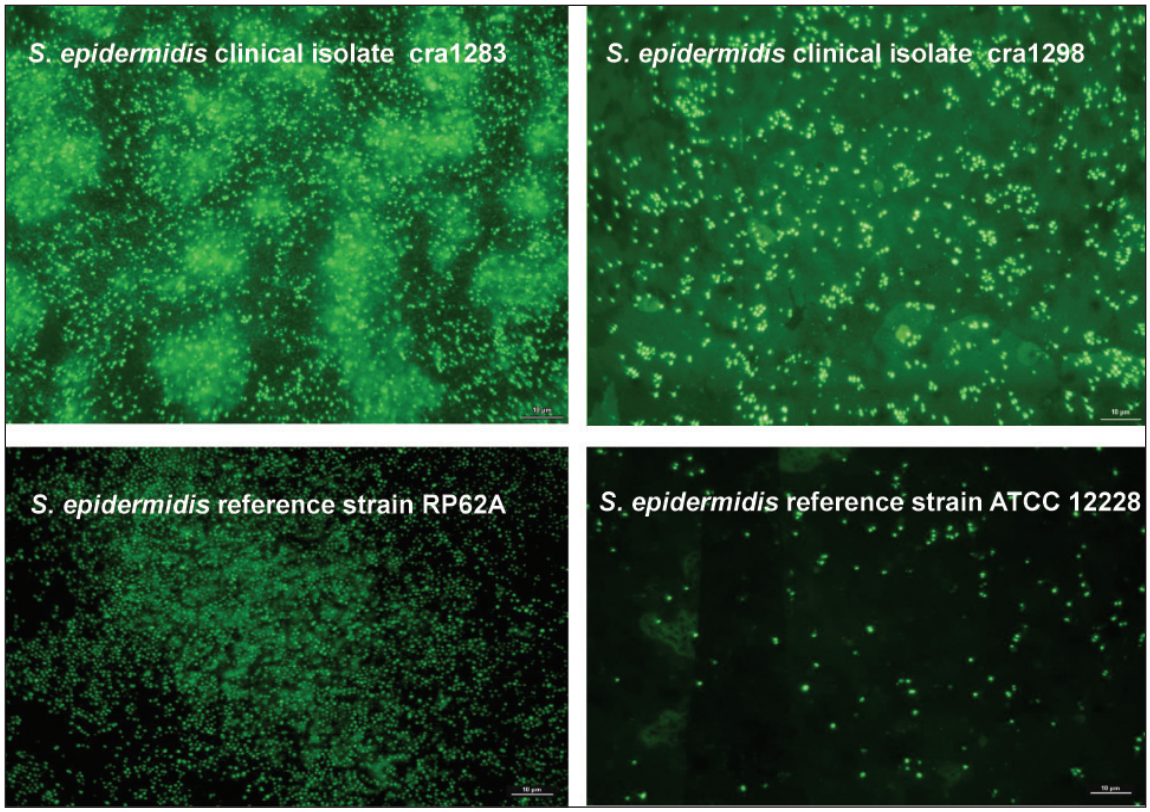

Fig. 1 - *Fluorescence microscopy of eDNA.*
A volume of 500 µL of the bacterial suspension of about 5 x 10^5 cells/ml was incubated in Chamber Slides (Thermo Scientific Nunc, Waltham MA, USA) for 72 hours at 37°C in static, humidified conditions. A volume of 250 µL of the bacterial suspension was replaced with an equivalent volume of fresh tryptose broth every 24 hours.
The bacterial suspension was taken off and the 72-hour-old biofilms were washed twice with 500 µL of Dulbecco's buffered saline solution (D-PBS). 250 µL of the SYTOX 2 µM solution were carefully applied directly on the glass surfaces of different chambers and incubated for 40 minutes, in the dark, at room temperature. The solutions were then taken off and excess staining solution was removed by washing two times with D-PBS; the plastic chambers were removed from the glass slides and these were washed once by immersion. The glass slides were left to dry at 37°C and then a drop of Vectashield® HardSet™ Mounting Medium (Vector Labs, Burlingame, CA, USA) was positioned on them. Coverslips were placed over the slides, and the slides were left overnight at 4°C and observed the day after.
The microscopic observations and image acquisition were performed with a Nikon Eclipse TE 300 fluorescence microscope (Nikon, Tokyo, Japan) equipped with detectors and filter sets (B-2A Filter Excitation: 450-490 nm, Medium Excitation Band / Emission: 515 nm, Longpass Emission Filter) for monitoring SYTOX (fluorescence excitation/emission maxima: 504 nm/523 nm when bound to DNA).
Images were obtained using a x100 oil objective. The bar represents 10 µm.

which has autolytic activity, induces lysis of a small fraction of the bacteria, resulting in the production of extracellular DNA which promotes surface attachment of the remaining population.

In Figure 1, personal observations on eDNA from two clinical isolates of *S. epidermidis* from implant infections are reported and compared with two reference strains (the biofilm-producing RP62A and the non biofilm-producing ATCC 12228). At 72 hours incubation abundant eDNA in clouds and flakes is evident in biofilm-producing *ica*-positive clinical isolate cra1283 and in the reference strain RP62A. In the cra1298 clinical isolate, a weak-biofilm-producing, *ica*-negative strain, many stained dead cells are seen, but the amount of stained eDNA is very scarce. A similar picture is observed with non biofilm-producing ATCC 12228 reference strain.

In the search of factors other than AtlE involved in DNA release and in biofilm formation, another autolysin, Aae, has been considered, but its role in DNA release has not yet been demonstrated (9).

The mechanisms of eDNA release by *Staphylococcus aureus* appear to be different. There is a general consensus

that in *S. aureus*, like in other species, eDNA originates from cell lysis and constitutes a necessary part of biofilm development. Unlike *S. epidermidis*, in which AtlE has a major role, in *S. aureus* the cell lysis depends on hydrolases controlled by the *cid* operon (10).

Two *S. aureus* operons, *lrgAB* and *cidABC,* regulate cell lysis and antibiotic tolerance in an opposing manner: the *lrg* operon decreases extracellular murein hydrolase activity and increases penicillin tolerance, whereas the *cid* operon increases extracellular murein hydrolase activity and decreases penicillin tolerance.

The *lrgA* and *cidA* gene products appear similar in structure and in function to the bacteriophage holin family of proteins, which are known regulators of murein hydrolase activity controlling the timing and onset of bacteriophage-induced cell lysis. Based on these similarities and on the phenotypic consequences of the *cid* and *lrg* mutations, it has been proposed that the *cidA* and *lrgA* gene products regulate murein hydrolase activity in a manner analogous to those of holins and anti holins, respectively, and thus they control cell death and lysis during biofilm development (11-13).

In fact, LrgA and CidA proteins are molecular control elements involved in the regulation of programmed cell death in *S. aureus*. In eukaryotic multicellular organisms and also in some unicellular eukaryotes (*Trypanosoma*, *Leishmania*, *Tetrahymena*), programmed cell death (PCD) is a genetically determined process of cellular suicide that is activated in response to cellular stress or damage, as well as in response to the developmental signals. Although historically studied in eukaryotes, it has been proposed that PCD also operates in prokaryotes, either during the bacterial life cycle or to remove damaged cells from a population in response to a wide variety of stresses (14, 15).

The study of Rice et al, has given evidence that in *S. aureus* CidA contributes to biofilm adherence both *in vitro* and *in vivo* by affecting cell lysis and the release of genomic DNA, highlighting the role of eDNA in *S. aureus* biofilm formation and the biological role of the Cid/Lrg system in this microorganism (10).

First demonstrated in *P. aeruginosa*, eDNA has been up to now described in a variety of bacterial species and its importance is recognized as a component of biofilm, which may contribute to the structural solidity of biofilms and to their recalcitrance to antibiotics by inducing expression of antibiotic resistance genes.

Structural role of eDNA: stabilization of the biofilm matrix

It is widely accepted that the presence of eDNA in biofilms has the important role of stabilizing their structure. Since 2002 in a pioneering study, Whitchurch et al (3) demonstrated that the formation of a stable biofilm and the attachment of bacterial cells to culture flow-chambers are prevented by the addition of DNase I to the culture medium. Moreover, established biofilms (up to 60 hours old) were dissolved by treatment with DNase I. Biofilms 84 hours old were more resistant to the DNase I treatment, suggesting the matrix in mature biofilms may be strengthened by substances other than eDNA or that mature biofilms may produce sufficient proteolytic exoenzymes to locally inactivate the DNase I.

Besides contributing greatly to understanding the mechanism by which eDNA is produced by *P. aeruginosa*, the study by Allesen-Holm et al provided a detailed description of the structural distribution of eDNA in the biofilm architecture (7). *P. aeruginosa* biofilms growing in flow chambers developed mushroom-shaped multicellular structures in a sequential process in which a stalk-forming sessile subpopulation was accompanied by a cap-forming migrating subpopulation.

By confocal laser scanning microscopy (CLSM), and the use of *P. aeruginosa* tagged with green fluorescent protein (Gfp) growing in flow chambers, the biofilm structures were studied after staining with DNA dyes that do not penetrate into live bacteria. The extracellular DNA was located primarily in the stalks of the mushroom-shaped multicellular structures, with a high concentration especially in the outer part of the stalks forming a border between the stalk-forming bacteria and the cap-forming bacteria.

Although eDNA has been found out to be a major structural component in many biofilms of *S. aureus*, its role and its structural organization remains enigmatic. Huseby et al have demonstrated that beta toxin, a neutral sphingomyelinase and a virulence factor of *S. aureus*, forms covalent cross-links to itself in the presence of DNA, producing an insoluble nucleoprotein matrix *in vitro* (16). They referred to this effect as biofilm ligase activity, independent of sphingomyelinase activity. Furthermore, they showed that beta toxin strongly stimulates biofilm formation *in vivo* as demonstrated by its role in infectious endocarditis in a rabbit model. The authors concluded that these results suggest that the cross-linking of beta toxin in the presence of eDNA has a role in forming the

skeletal framework upon which staphylococcal biofilms are established.

An intriguing issue, raised by the authors themselves, is the role of *agr* on beta-toxin production on the one hand and on biofilm formation on the other. Beta toxin production, like that of shock syndrome toxin-1, is positively regulated by signals of the *agr* system, whereas silencing of *agr*, by mutation, has the effect of stimulating biofilm formation. Beta toxin expression should be then accompanied by thin biofilms, rather than what they effectively present. The authors refer to the observations by Abdelnour et al (17) and Xiong et al (18) that persistent staphylococcal infections become *agr*-negative over time. However, as the authors admit, chronic infections require expression of toxic shock syndrome toxin-1 (and presumably beta toxin). Therefore, as the authors conclude, the role of beta-toxin in DNA stabilization of biofilm requires further studies.

The crucial importance of eDNA in biofilm settling and stabilization is in any case highlighted by an *ex juvantibus* criterion: the disgregating effect of DNase I on biofilm. As recalled above, DNase I is able to inhibit biofilm formation when present in the culture medium at the time of seeding bacteria, but when biofilm has grown in the absence of DNase I, the enzyme appears less effective in disrupting formed biofilm.

Two possible mechanisms for the DNase I inhibition of biofilm formation have been considered. The enzyme could dissolve nucleic acids associated with bacterial surface acting as an adhesin which promotes initial attachment to biomaterial surfaces. Alternatively, the enzyme could degrade eDNA, which especially in young biofilms, is the principal cell-to-cell adhesin able to form a filamentous mass surrounding and interconnecting aggregates of bacterial cells (19). Beside inhibiting biofilm formation, DNase I has also been shown to sensitize biofilm bacteria to the killing action by various biocides and also to detachment by the anionic detergent (20, 21). According to Kaplan, DNase I-mediated biofilm detachment may occur rapidly and at clinically achievable concentrations of the enzyme, making it a candidate for therapeutic action (22).

Functional role of eDNA: part of gene-transfer mechanisms

Besides participating in biofilm development and in the stabilization of biofilm structure, released eDNA has an important part in gene-transfer mechanisms. This genetic transfer creates an opportunity for a rapid spreading of virulence as well as antibiotic resistance genes in circulating strains exposed to the selective pressure of medical treatments.

Extracellular DNA present in bacterial communities organized in biofilms constitutes a dynamic gene pool from which bacteria competent for natural transformation can derive genetic information by horizontal gene transfer, a process by which genetic information is passed from one bacterial genome to another. The impact of horizontal gene transfer is exemplified by bacterial acquisition of virulence traits and antimicrobial drug resistance. One example of bacteria that are capable of exchanging genes is *Streptococcus pneumonia* and related streptococci.

Competence is a physiological state in which bacteria develop a capacity to take up exogenous DNA. This intrinsic property is not constitutive but is regulated by a secreted competence-stimulating peptide (CSP) and its cognate signal transduction pathway (23). In *Streptococcus pneumoniae,* when CSP reaches a threshold of 1 ng/ml to 10 ng/ml in the medium, a subpopulation of the bacteria lyse, and the released DNA is taken up by the remaining population (24). The CSP system has been shown to contribute to biofilm formation in several species of Streptococcus (25-27). Biofilms produced by competence-defective mutants have reduced biomass; conversely, in Streptococcus intermedius and Streptococcus mutans the addition of synthetic CSP increases competence and accelerates the growth of biofilms (27-28).

Exogenous DNA represents an important mechanism for horizontal gene transfer in naturally competent bacteria (29). Since bacterial population in a biofilm is essentially homologous, the final utility to transfer genetic information through the population is not immediately evident. Spoering and Gilmore (30) have argued that the uptake of DNA from sibling cells might have the same benefit for recombinational purpose as that achieved by sexual reproduction in eukaryotes, in that the DNA uptake increases the chance that some beneficial mutation, independently arisen, can accumulate in the bacterial population.

Emergence and spread of antibiotic resistance by horizontal gene transfer may be a dreadful consequence of eDNA released in a biofilm. Acquired antibiotic resistance can emerge from susceptible bacterial isolates either by mutation or by acquisition of resistance genes. But, while the proportion of mutant cells in a bacterial population is usually

very low (10^{-8} - 10^{-9}), the propagation of resistance genes may be greatly favored not only by positive selection of rare mutants during prolonged antimicrobial treatment but also by the release of eDNA by mutants and by the assumption of DNA molecules by sibling bacterial cells. Prolonged antimicrobial treatment now and again exerts a positive selection on the cells which have acquired resistance determinants by horizontal gene transfer, expanding resistant bacteria in biofilm (31). Biofilm production, with its high cell density, and eDNA release synergistically contribute to the emergence and spreading of antibiotic resistance by horizontal gene transfer.

Role of eDNA in conditioning innate immune response, prevention of phagocytosis, and attenuation of inflammation

As outlined above, bacterial biofilms, including those formed by *S. aureus*, are encased in a complex matrix composed of polysaccharides, extracellular DNA (eDNA), and proteins. All these microbial structural motifs are recognized by the innate immune system via the TLR family of pattern recognition receptors (PRRs) (32).

TLR2 mediates recognition of several pathogen-associated molecular patterns expressed by *S. aureus* that are released during normal bacterial growth and lysis, including lipoproteins, peptidoglycan (PGN), and lipoteichoic acids. TLR2 are expressed by numerous cell types, macrophages, dendritic cells, and phagocytosis of *S. aureus* is required to trigger TLR2-dependent signaling within the phagosome and inflammasome activation. TLR9 is an intracellular receptor that recognizes unmethylated CpG motifs characteristic of bacterial DNA (33, 34). Upon phagocytosis and digestion of *S. aureus* in the phagosome, bacterial DNA is liberated and engages TLR9. TLR9-dependent activation can be triggered not only by phagocytosis of whole *S. aureus* cells but also by that of extracellular DNA, extensively contained in the biofilm matrix. The role of TLR2 and TLR9 in regulating host immunity to *S. aureus* biofilm growth has been examined by Thurlow et al (35). Although both TLRs are pivotal for host immune responses to planktonic *S. aureus* and associated pathogen-associated molecular patterns, the behavior of immune response appears different in *S. aureus* biofilm infections. Using a mouse model of catheter-associated biofilm infection, they demonstrate that *S. aureus* biofilms actively attenuate traditional antibacterial immune responses, as demonstrated by significant reductions in cytokine/chemokine production associated with biofilm-infected tissues compared with the wound healing response elicited by sterile catheters. Although macrophage infiltration into biofilm infections was prominent, immunofluorescence staining revealed that few cells were able to migrate into the biofilm. Significant and rapid cell death in macrophages that invaded deeply into the biofilm was observed. Thurlow et al concluded that *S. aureus* biofilms are able to circumvent traditional antimicrobial effector pathways and persist in an immuno-competent host. Thurlow's study demonstrates that *S. aureus* attenuates inflammatory mediator production and macrophage invasion into the biofilm.

Intracellular invasion, impairment of phagocytes and, above all, shielding by biofilm protect staphylococci from innate and adaptive immune responses. They also enhance resistance to antibiotics and sustain the persistence of implant infections by *S. aureus* and the irreducibility of implant infections caused by staphylococci growth. The relationship between eDNA release and biofilm production by staphylococci on the one hand and innate immune responses and phagocytosis on the other hand have been recently presented and discussed in last two issues of the IJAO Focus on Implant Infections (36-39).

Molecular studies on biofilm composition, on the mechanisms of eDNA release, and on gene transfer in biofilms, besides enriching the initial acquisitions (40-42) on the role of PIA and *ica* locus in the pathophysiology of biofilm and in pathogenesis of implant infections, are also prompting advances in measures for controlling biofilms. Ongoing spin-offs that are broadening our knowledge on biofilm-related infections include the use of enzymes able to disrupt biofilm structure in association with antibacterial agents, caution in the use of antibiotics, progress in stimulating immune responses and in controlling inflammation responses and, finally, introduction of new anti-biofilm implant materials (43-52).

Conflict of Interest Statement: The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Address for correspondence:
Professor Carla Renata Arciola
Head of the Research Unit on Implant Infections
Rizzoli Orthopaedic Institute
Via di Barbiano 1/10
40136 Bologna, Italy
e-mail: carlarenata.arciola@ior.it

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