Opinion

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Initiation of Infection

Biofilm switch and immune response determinants at early stages of infection

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Biofilm development is recognized as a major virulence factor underlying most chronic bacterial infections. When a biofilm community is established, planktonic cells growing in the surroundings of a tissue switch to a sessile lifestyle and start producing a biofilm matrix. The initial steps of *in vivo* biofilm development are poorly characterized and difficult to assess experimentally. A great amount of *in vitro* evidence has shown that accumulation of high levels of cyclic dinucleotides (c-di-NMPs) is the most prevalent hallmark governing the initiation of biofilm development by bacteria. As mentioned above, recent studies also link detection of c-di-NMPs by host cells with the activation of a type I interferon immune response against bacterial infections. We discuss here c-di-NMP signaling and the host immune response in the context of the initial steps of *in vivo* biofilm development.

Uncertainties in the initiation of biofilm formation in chronic infections

Bacterial infections in the human body are classically divided into acute and chronic infections. This useful clinical classification usually reflects differences in the expression of gene products known as virulence factors as well as in the lifestyle that the bacteria causing the infection adopt. Acute infections last a short time and involve single planktonic (free-swimming) bacteria that cause tissue damage through the production of a large amount of virulence factors. They typically cause severe clinical symptoms and can generally be treated efficiently with one or more antibiotics. By contrast, chronic infections are characteristically associated with bacterial aggregates, commonly referred to as biofilms, where bacteria are physically associated and encased in a self-produced extracellular matrix composed of exopolysaccharides, large surface proteins, fatty acids, and DNA. Biofilm-mediated infections persist despite antibiotic therapy and the host innate and adaptive immune responses [1–4]. Although the biofilm is the predominant mode of growth for bacteria in most natural and clinical environments, some bacterial species are more prone to produce chronic infections, suggesting that they either have a high capacity to reach locations suitable for biofilm settlement or possess special skills to switch efficiently from a planktonic to a sessile lifestyle on the surface of inert or living tissues. Despite the wealth of knowledge gained over the years on biofilm formation and its implication in chronic infections [5,6], very little is known about how the biofilm development process starts on the surface of host tissues and how the host cell responds to this process. In this regard, the animal models currently used to examine *in vivo* biofilm infections, such as device-related endocarditis or placement of a piece of catheter under the skin, provide insights into the factors required for the progression of the infection. However, new methodological strategies, such as direct observation of biofilm development on tissue surfaces and *in situ* genome-wide transcriptomic profiling, will be necessary to explore further the initial steps governing *in vivo* biofilm development and the progression of chronic infection. Significant challenges for the implementation of these methods are the low number of planktonic bacteria from which biofilm infection is thought to start and also the short time-period between initial bacterial adhesion and the switch to a sessile lifestyle. Thus, taking into consideration the scarcity of background information, we first provide a brief overview of the main signal transduction pathway, based on c-di-NMP secondary messengers, that governs the initial planktonic-to-biofilm switch, and then discuss the emerging information about the role of c-di-NMPs as pathogen-associated molecular patterns (PAMPs) triggering a host innate immune response. Subsequently, we raise the possibility that both processes might be connected during the initial steps of biofilm development that precede the establishment of a chronic infection.

Switch to biofilm formation and signal transduction

A first step triggering the transition between a motile, single cell state to an adhesive, multicellular state on the surface of a particular tissue is the recognition and transmission of signals that bacteria use to determine if settlement is appropriate. Several signals have been shown to favor early settlement of bacteria on human tissues including: (i) the presence of inert surfaces (plastics, metals, and devitalized bone); (ii) increased amounts of extracellular

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iron and ferritin that induce a *Pseudomonas aeruginosa* biofilm phenotype in the sputum of cystic fibrosis patients; (iii) compromised tissues with elevated levels of glucose in diabetic patients; (iv) imbalances in the commensal microbial communities caused by antibiotic treatments or age-related deterioration; (v) simultaneous or a previous viral infection; (vi) hydrogen peroxide produced by neutrophils during the oxidative burst; (vii) the presence of compounds such as indole that has stimulating effects on biofilm formation by many Gram-negative bacteria including *Escherichia coli*, *Klebsiella oxytoca*, *Citrobacter koseri*, and *Haemophilus influenza*; other compounds include extracellular polyamines, calcium, and bile salts that modulate biofilm formation in *Vibrio cholerae*, *Yersinia pestis*, *Pseudomonas putida*, and *Staphylococcus aureus* [7–12]. Bacteria have two major sensory systems for the recognition of these signals: two-component systems (TCSs) and the c-di-GMP-mediated signal transduction network. TCSs provide the basic stimulus–response coupling mechanism in bacteria. The best-characterized example of a TCS driving the motile–sessile switch is the intricate LadS/RetS/Gac/Rsm signal transduction system of *P. aeruginosa*, where the GacS/GacA TCS represses the expression of the CsrA homolog RsmA, which reciprocally regulates factors involved in acute infection (motility and the type III secretion system) and chronic infection (exopolysaccharides and the type VI secretion system) ([13,14] for review). However, TCSs are not typically implicated in controlling the switch between planktonic and sessile lifestyles. Instead, they are dedicated to matching the production of specific compounds of the biofilm matrix to the environmental conditions. The signal transduction system responsible for inhibiting motility and promoting biofilm development in a diverse range of bacterial species depends upon the secondary messenger c-di-GMP. In c-di-GMP signaling, the sensor protein domain reacts to the stimulus by activating an output domain located in the same protein that triggers the synthesis [diguanylate cyclase (DGC), GGDEF domain-containing proteins] or degradation [phosphodiesterase (PDE), EAL and HD-GYP domain-containing proteins] of c-di-GMP. Then, the resulting c-di-GMP interacts with specific effectors that finally relay the signals to cellular processes [15]. Based on *in vitro* studies carried out with different bacterial species, it is widely accepted that a high concentration of c-di-GMP enhances biofilm development and represses virulence factor expression, whereas low c-di-GMP levels promote bacterial motility and a planktonic lifestyle [16–19]. If this concept is extrapolated to what should happen when bacteria start producing a biofilm on the surface of a host tissue, the switch from a planktonic to a biofilm lifestyle will also depend on the accumulation of c-di-GMP through the activation of specific GGDEF domain-containing proteins or the inhibition of EAL/HD-GYP domain proteins. Because very often bacteria contain several GGDEF and EAL/HD-GYP domains linked to signal input domains [including PAS, REC, globin, blue light sensing (BLUF), hemerythrin, GAF, CHASE, and MASE domains], it is conceivable that one or more of these proteins sense specific signals on the surface of host tissues, either upon surface contact or as a prerequisite for attachment, to promote increased c-di-GMP accumulation [20]. Unfortunately, to date there is very little information about the mechanisms that regulate DGC and PDE activities *in vivo*, and few studies have been able to show a role of c-di-GMP during chronic infections. For example, small-colony variants of *P. aeruginosa* with elevated c-di-GMP levels have been identified in cystic fibrosis sputum samples [21–23]. In addition, Byrd *et al.* were able to show that *P. aeruginosa* c-di-GMP overproduction mutants had increased persistence as compared to the wild type strain in a chinchilla middle-ear infection model [24]. Considering that there is a general agreement that c-di-GMP signaling is determinant for biofilm development, why has the analysis of c-di-GMP contribution to *in vivo* infections (and particularly to the initial steps of infection) trailed behind? Detection of c-di-GMP is technically very challenging and current methods cannot be applied to measure c-di-GMP during infection. In this regard, the development of a highly-sensitive c-di-GMP-dependent reporter system to monitor rapidly the level of the nucleotide in infecting living bacteria would be extremely useful for investigating the role of this signaling pathway during infection. Because few c-di-GMP receptors have been identified, the experimental strategy to characterize the role of c-di-GMP has been based on the analysis of bacterial mutants overloaded with, or depleted, of c-di-GMP. This is in principle a valid approach that has produced many important insights, but a serious problem is that constant high or low levels of c-di-GMP may affect the activity of several c-di-GMP receptors at the same time, and thus can influence more than one bacterial process. This scenario may not necessarily resemble a real progression in the establishment of a chronic infection. In support of this prediction, the pioneering work of Pultz *et al.* [25] has shown differences in the affinities for c-di-GMP of receptor proteins involved in the coordinated inhibition of bacterial motility and increased matrix synthesis during *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) biofilm formation. Thus, this model involves the sequential activation of c-di-GMP receptors such that, if the c-di-GMP concentration is maintained at a low level, the bacteria are motile and do not produce biofilm matrix compounds. Upon sensing appropriate environmental conditions, levels of c-di-GMP rise, first activating the receptor responsible for flagella motility repression, and later the receptor that stimulates the synthesis of biofilm matrix compounds. This model implies that when a *S.* Typhimurium mutant with high levels of c-di-GMP is used for virulence studies it is necessary to consider that the resulting phenotypes may be due to both the inhibition of bacterial motility and shielding of cell surface receptors by biofilm matrix compounds, and this may not mimic the reality of sequential biofilm formation events *in vivo*.

Despite the broad distribution of c-di-GMP signaling in bacteria, some bacteria lack the enzymes required for c-di-GMP synthesis and instead they use c-di-AMP as a secondary messenger [26–30]. A notable example is *S. aureus*, one of the bacterial species most frequently associated with biofilm-mediated infections [31,32]. Although our knowledge about c-di-AMP-mediated signaling in bacteria is in its infancy compared to the c-di-GMP network, it is already clear that c-di-AMP controls cell wall properties because an