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The polymicrobial nature of biofilm infection

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Abstract

The model of biofilm infection was first proposed over a decade ago. Recent scientific advances have added much to our understanding of biofilms, usually polymicrobial communities, which are commonly associated with chronic infection. Metagenomics has demonstrated that bacteria pursuing a biofilm strategy possess many mechanisms for encouraging diversity. By including multiple bacterial and/or fungal species in a single community, biofilms obtain numerous advantages, such as passive resistance, metabolic cooperation, byproduct influence, quorum sensing systems, an enlarged gene pool with more efficient DNA sharing, and many other synergies, which give them a competitive advantage. Routine clinical cultures are ill-suited for evaluating polymicrobial infections. DNA methods utilizing PCR methods, PCR/mass spectroscopy and sequencing have demonstrated their ability to identify microorganisms and quantitate their contribution to biofilms in clinical infections. A more robust model of biofilm infection along with more accurate diagnosis is rapidly translating into improved clinical outcomes.

Keywords: Biofilm, co-aggregation, horizontal gene transfer, metabolic cooperation, passive resistance, PCR, polymicrobial, sequencing, synergies

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Introduction

It has been over a decade since Costerton and Stewart [1] proposed a simple model of biofilm infection.The evolving biofilm infection paradigm was a significant departure from the then widely held view of infection, which envisioned single-species bacteria in a planktonic mode of growth utilizing virulence factors to cause infection [2,3]. This planktonic view of infection could explain most acute infections, but was wholly inadequate for understanding chronic infections. However, Costerton and Stewart's early innovative biofilm model of infection demonstrated, at the biochemical and cellular levels, a new bacterial strategy by which communities of bacteria produce infection.

Their biofilm model of infection explained the ineffectiveness of antibodies [4] and white blood cells [5] in combating biofilms. Other work showed the ineffectiveness of antibiotics for clearing a biofilm infection [6]. The final thread was the biofilm's ability to induce host hyperinflammation, as shown by elevated levels of proinflammatory cytokines [7] and matrix metalloproteases [8], and excessive numbers of neutrophils [9].

Over the last decade, many new studies utilizing an emerging and sophisticated science have generated a wealth of fresh insights into the nature of biofilm infection. It is hoped that, through weaving of these different threads of new information into the original biofilm model of infection, a robust tapestry will emerge that will allow for more focused and productive research going forward.

Metagenome

Kim [10], in a recent review of the molecular pathways that bacteria utilize for producing host infection, found that these molecular pathways could be grouped into two different types. One group of mechanisms was clearly for breaching

the host tissue, producing cell death and necrosis for bacterial nutrition. The other group comprised molecular mechanisms by which bacteria could attach to host cells, and inject small effector proteins that commandeered host cellular pathways to reorganize the cellular cytoskeleton [11,12], prevent migration [13], prevent mitosis [14] and, most importantly, inhibit apoptosis [15–18]. This 'new' model of infection encompasses the molecular strategies employed by biofilm phenotype bacteria.

Bacteria pursuing a biofilm strategy for infection have molecular mechanisms for recruiting other bacteria. It seems that biofilms actively attempt to become polymicrobial, apparently to improve their survivability. There has been a shift in microbiology into thinking of biofilms as systems with global regulation of the expanded gene pool provided by species diversity [19]. This new understanding suggests that a biofilm is a single entity that exerts central control over the individual members to yield the activities necessary for the colony's survival. Biofilms require gene expression that allows for attachment to the host, produces host cellular senescence to prevent shedding, and causes local inflammation that creates plasma exudate for sustained colony nutrition [20]. Microorganisms may combine their genetic resources to fulfil these requirements, so that each individual member of a biofilm need not possess all of the genes necessary to carry out each function. This has led to the proposal of functional equivalent pathogroups, which are frequently identified recurring groups of microorganisms in biofilm infections [21].

It has been demonstrated that, in *Streptococcus pneumoniae*, individual members of the community possess only a proportion of all the genes present within the culture, and this has led to the distributive genome hypothesis [3,22]. Sharing the total genes of the species (supragenome) allows each member to expend less energy in maintaining its proportion of the total gene pool, while allowing the entire community to have all of the genes present [23]. Application of this principle to polymicrobial biofilms has led to the suggestion that the genomic plurality leads to the continuous production of novel strains, fostering a persistent infection [24]. The main molecular method by which genomic plurality is accomplished within the biofilm is upregulated and highly efficient horizontal gene transfer [25,26].

In clearly defined spatial locations within the biofilm, horizontal gene transfer is optimized by quorum sensing systems, and is usually much more efficient than the planktonic phenotype. Horizontal gene transfer, much more than vertical gene transfer from the parent cell to the offspring, has been reported to be the main mechanism for distributing genes within prokaryotic bacteria [27,28]. The close spatial arrangement of the community, along with quorum sensing, allows for more efficient DNA transfer among the members, mainly through conjugation, occasionally through transformation, and rarely through bacteriophage-mediated transduction [28]. Horizontal gene transfer is more efficient in permissive regions of the biofilm and severely limited in regions of low/no growth, owing to the accumulation of metabolic byproducts [29].

It has long been known that species diversity in ecological systems provides greater ability to withstand various stresses; this is known as the 'insurance hypothesis' [30]. Boles's group [31] took this one step further, showing that *Pseudomonas aeruginosa*, through a *rec*A-dependent mechanism, self-diversifies its gene pool to become more recalcitrant in an infection. So, whether by functional equivalence, a distributive genome, self-diversification, or other methods, biofilms seek to expand their genetic diversity in order to 'insure' survival.

Synergies

Biofilm communities in most environments, including human disease, tend to be polymicrobial [32]. By including multiple bacterial and/or fungal species in a single community, biofilms obtain numerous advantages, such as passive resistance [33], metabolic cooperation [34,35], byproduct influence [36], quorum sensing systems [34], an enlarged gene pool with more efficient DNA sharing [37], and many other synergies, which give them a competitive advantage. It is best to view a biofilm as a single entity possessing multiple genetic resources that allow it to adapt and thrive regardless of the stresses that it encounters. In general, the greater the diversity, i.e. the larger the gene pool, the more robust the biofilm is in terms of its survivability [38].

Individual bacteria possess multiple molecular mechanisms to actively co-aggregate with other beneficial species. Co-aggregation mechanisms are usually reversible molecular bonds that allow the genetically distinct bacteria to select for beneficial partners within the biofilm [39]. Co-localization is a similar concept, but carries the connotation of being a more passive process. In co-localization, a species of bacteria will encourage the local growth of a beneficial partner by providing benefits for its growth rather than by utilizing physical bonds.

Unique species of microorganisms that have the ability to form biofilms usually possess species-specific quorum sensing molecules to direct the organization of the monoclonal biofilm. For polymicrobial biofilms, there are some quorum sensing molecules that can upregulate pathways in multiple

species that are necessary for the individual constituents to cooperate within the community. Autoinducer 2 is a small boron-containing quorum sensing molecule that can be recognized by many different species of bacteria, including anaerobes, Gram-negative bacteria, and Gram-positive bacteria [40]. Autoinducer 2 has been shown to be present in wound biofilms, as well as in other chronic infections [41]. Similarly, it has been demonstrated that bacteria respond to host molecules, such as adrenaline [42,43], and there is growing evidence of bi-directional inter-kingdom signalling [44]. The organisms in biofilm infections possess the ability to sense and respond to their neighbours and to their host through multiple quorum sensing pathways.

Metabolic cross-feeding between genetically distinct species has been well established. It has been shown that *Streptococcus gordonii* produces peroxide that can cause *Aggregatibacter actinomycetemcomitans* to produce a factor H-binding protein that limits the host's ability to kill *A. actinomycetemcomitans* through complement-mediated lysis [45]. *A. actinomycetemcomitans* can utilize the lactic acid byproduct of streptococcal metabolism, which not only benefits *Streptococcus* but also enhances the virulence of *A. actinomycetemcomitans* [46]. This metabolic cooperation has been identified in numerous polymicrobial models [47–49].

The most common form of nutrient depletion within biofilms is the development of an anoxic region within the core of the biofilm [50]. *In vitro* biofilm models have been developed to exploit this property of biofilms, to allow the propagation of anaerobic bacteria in what is viewed as an oxygen-rich environment, such as the surface of a chronic wound [51]. Also, *in vivo* polymicrobial biofilm models now allow the evaluation of hypoxia, pH, and species interactions [48]. The ability of biofilms to provide an environment for anaerobic growth through oxygen depletion may be an important factor in the increase in the virulence of biofilm infections [52–54].

Waste products, molecules that bacteria produce which are end-products and are of no benefit to the metabolizing member, are released into the local biofilm environment. Many of these metabolites, such as ammonia, lactic acid, and carbon dioxide, can have significant influences on the surrounding microorganisms [36]. Studies have demonstrated that *Fusobacterium nucleatum* and *Prevotella intermedia* generate ammonia, which raises the pH to a level suitable for *Porphyromonas gingivalis* [55], and that *F. nucleatum* also increases the level of carbon dioxide, which increases the pathogenicity of *P. gingivalis* [56]. Also, it has been shown that waste products from *Pseudomonas aeruginosa* may protect *Staphylococcus aureus* from aminoglycosides [57].

Passive resistance occurs when one of the members in the biofilm possesses a resistance factor that can protect other members of the biofilm that do not have the factor. There are numerous biofilm defences that limit the effectiveness of antibiotics. However, the easy sharing of mobile genetic elements, such as *mec*A cassettes and genes encoding extended-spectrum β-lactamases, raises concern about increasing passive resistance in polymicrobial infections. For example, a β-lactamase-producing strain of *Haemophilus influenza* was co-cultured with *S. pneumoniae* lacking any resistance factors. *H. influenza* increased the MIC/MBC of amoxycillin for *S. pneumoniae* [33].

The clinical concern about the synergies of polymicrobial biofilms is that the infection will be more severe and recalcitrant to treatment. There are many examples showing that this is indeed the case. Low levels of *Pseudomonas aeruginosa* mixed with *Staphylococcus aureus* increased infection rates in a rat model [58]. In a mouse model, *Prevotella* increases the pathogenicity of *Staphylococcus aureus* [59]. *Escherichia coli* produced a marked increase in the size of abscess formation with *Bacteroides fragilis* in a diabetic mouse model [60]. There is also clinical evidence suggesting that polymicrobial infections are more severe [38].

Diagnosing Polymicrobial Communities

The clinical significance of shared genetic information, metabolic synergies and co-aggregation/co-localization symbiosis is that new diagnostic and therapeutic methods will be required. Because, as discussed above, minor bacterial constituents can provide a multitude of different advantages to their neighbours, including increased virulence, it is important to identify all of the species present and their relative contributions to the infection. Clinical diagnostic methodologies are focused on identifying the most abundant organisms producing an infection, but for biofilms this will have to give way to methods that can identify and quantitate each individual member of the polymicrobial community. Molecular methods have demonstrated the ability to precisely define the identity and quantity of each species of a polymicrobial biofilm infection [62–67].

The 16S rDNA has been called the genomic fingerprint, and molecular methods (PCR and sequencing) evaluating the 16S rDNA region are usually capable of reading this genomic fingerprint [62]. PCR methods are more suited to looking at unique genetic targets within a particular species, but are also used to evaluate the 16S rDNA [62]. However, the specificity of PCR is a limitation, in that microorganisms can only be identified if a specific primer is developed and validated. Because there are thousands of different species present in biofilm infections, this is currently impractical. To

avoid the necessity of developing an almost infinite number of primers, innovators have integrated PCR of the bacterial 16S rDNA gene with rapid evaluation of this gene by mass spectroscopy [68]. The Plex ID (Abbott Molecular) utilizes multiple general primer sets to cover the different variable regions of the 16S rDNA as well as other chromosomal and plasmid regions, so that the vast majority of the currently reported bacterial species can be identified [69]. By the use of advanced mathematical algorithms, segments of the 16S rDNA can be correlated with specific species with a very high level of confidence. This allows rapid same-day identification and some quantitation of the more prevalent organisms within a polymicrobial infection [68].

Sequencing technologies such as pyrosequencing based on a light signal (Roche 454), PacBio based on kinetic properties (Pacific Biosciences) and ion torrent based on pH signals (Life Technologies) use different methods, but they all accurately determine the sequences of long segments of specific regions of bacterial DNA, such as the 16S rDNA gene. These technologies can give a 99% accurate code for the 16S rDNA gene that is then compared against accessible databases to identify the microorganism with a high degree of certainty. The copy number for each specific organism can be compared with the total number of copies for all organisms, providing good relative quantification for each microbial species present [70].

An issue is whether the bacterial DNA identified is from bacteria that are alive. Ehrlich showed that 'These findings indicate that purified DNA and DNA from intact but nonviable bacteria do not persist in the middle ear cleft in the presence of an effusion' [71]. It therefore seems likely that the majority of organisms identified by their DNA are indeed viable organisms.

Molecular methods, even at this early stage, can handle the challenges of correctly diagnosing polymicrobial infection. Once good DNA is obtained, most molecular platforms are extremely capable of accomplishing their analysis. However, cleaning up of the data post-analysis, such as chimera checking, noise suppression and data housekeeping after the run, is critical. Reporting the identification and amounts of the organisms present requires complex bioinformatics to reduce the massive amounts of data down to reliable, clinically useful results. However, even with all of these technical hurdles, molecular methods have demonstrated the unique ability to reliably evaluate biofilm infection [21,52,61,65,66].

Conclusion

Over 17 million people in the USA develop a chronic infection annually, and *c.* 500 000 people die with or from a chronic infection each year [72]. This underscores the urgent need for a deeper understanding of polymicrobial infection. The amount and diversity of genetic material available to a polymicrobial infection strongly influence the severity and recalcitrance of the infection. Also, the interactions between the available genes, competitive and/or synergistic, may provide important therapeutic targets in the near future. Therefore, determining the species present and their relative contributions to biofilms, to understand and to exploit these targets, is of great clinical importance. Far from being reductionist, by breaking biofilms into their parts, molecular diagnostics provide a valuable clinical tool with which to truly comprehend the mobile genetic elements, genes and synergies that determine the behaviour of the unique biofilm producing a polymicrobial infection.

Transparency Declaration

Conflict of interest: RW an equity owner in pathogenies Laboratory.

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