Molecular Mechanisms of Biofilm Infection: Biofilm Virulence Factors

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Background: Numerous planktonic virulence factors have been identified that enable bacteria to successfully produce acute infections in tissues. In contrast, very little is known about biofilm virulence factors that enhance the establishment and long-term survival of biofilms in tissues.

The Problem: There is a need to identify the genes that encode biofilm virulence factors and understand how these factors function to enable bacteria to successfully establish chronic biofilm infections in tissues.

Basic Science Advances: A methodology was developed to screen 6,912 *Pseudomonas aeruginosa* loss-of-function mutants in a murine model of airway infection to determine which genes were crucial for establishing chronic lung infections in mice. Genetic analysis of 16 bacterial mutants isolated using this methodology identified 15 separate biofilm virulence factor genes whose loss-of-function increased biofilm survival. Sequence analysis of clinical Isolates obtained from seven cystic fibrosis (CF) patients with chronic infections that were collected over 16.3 years identified loss-of-function mutants for 7 of these 15 genes.

Clinical Care Relevance: Identifying biofilm virulence factor genes further defines the molecular mechanisms of establishing chronic biofilm infection and should lead to more effective and specific treatments to prevent biofilm formation and/or improve clearance of chronic biofilms in patients.

Conclusion: Biofilm virulence factor genes were identified in *P. aeruginosa* using an animal model of chronic lung infection for mutant screening and from infected CF patient isolates. In many cases they were inactivated planktonic virulence factor genes. These results further demonstrate the opposing patterns of gene expression between acute planktonic bacterial infections and chronic biofilm infections.

BACKGROUND

A COMPREHENSIVE VISION for biofilm infection was introduced by Costerton and Stewart in their seminal article in *Science* in 1999.¹ They suggested that biofilm infections in a host environment might start with planktonic bacteria attaching to exposed epitopes (lesions) in an impaired host environment. During metamorphosis to a biofilm phenotype, extracellular polymeric substances (EPS) are secreted around

the biofilm bacteria, firmly attaching the bacteria to the host surface and providing protection from host immunity. Once a quorum (minimum number) is established, through recruiting other bacteria or through cell division, quorum-sensing pathways are dynamically regulated, leading to phenotypic changes in discrete regions producing the functional colony components that lead to mature biofilm.² The Costerton-Stewart proposition of biofilm infec-

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Abbreviations and Acronyms

CF = cystic fibrosis
EPS = extracellular polymeric substance
IL-8 = interleukin 8
LOS = lipooligosaccharide
NET = neutrophil extracellular trap
NTHI = nontypeable *Haemophilus influenza*
OD = optical density
Pos-STM = positive signature-tagged mutagenesis
SCV = small-colony variant

tion¹ provided a sufficient understanding of the experimental evidence to that time, but the model lacked the molecular underpinnings to validate it as a scientific theory. Serendipitously, most of the molecular information necessary to validate the theory of biofilm infection has been uncovered during more recent studies, many of which were designed on a planktonic paradigm. Molecular mechanisms that have been shown to enhance establishment of bacterial biofilm indicate that biofilm virulence factors likely arise through adaptive mutations and favor defensive rather than invading/breaching behavior, resulting in maintenance of the host niche.

TARGET ARTICLE

Bianconi I, Milani A, Cigana C, Paroni M, Levesque RC, Bertoni G, and Bragonzi A: Positive signature-tagged mutagenesis in *Pseudomonas aeruginosa*: tracking patho-adaptive mutations promoting airways chronic infection. PLoS Pathog 2011; 7: e1001270.

CLINICAL PROBLEM ADDRESSED

In planktonic infections (*i.e.*, acute infection), the expression of planktonic virulence factors favors bacterial mechanisms that facilitate invasion and use of host tissues/cells as a nutrient source. In biofilm infection, the host niche can be maintained for years, even decades such as in otitis media, osteomyelitis, endocarditis, cystic fibrosis (CF), and periodontitis. Understanding the molecular mechanisms of chronic biofilm infection, particularly the identification of biofilm virulence factor genes, could be used to improve clinical assessment and may eventually lead to identification of chronic biofilm infection factor profiles (signatures) that can be used to predict the relative risk of clinical outcomes.

RELEVANT BASIC SCIENCE CONTENT

In the field of bacterial infection, a great deal of basic research has focused on molecular mechanisms that both pathogenic and commensal bacteria employ to establish an infection in the host and persist. The molecular mechanisms by which bacteria establish their initial foothold in a host environment are many. However, it is still unclear at what point the biofilm phenotype becomes relevant. The first line of defense against acute bacterial infection involves the recruitment and sequestration of neutrophils by the chemokine in-

terleukin 8 (IL-8).³ Recent studies have shown that neutrophils employ a variety of molecular mechanisms in response to bacterial infection (*e.g.*, phagocytosis, neutrophil extracellular traps [NETs], antimicrobial peptides also called host defense peptides, etc.).^{3–7} Likewise, bacteria employ a wide variety of molecular mechanisms to evade or suppress neutrophil activity.^{4–6} Depending on the bacterial species, these molecular mechanisms may result in establishment of a hyper-inflammatory milieu resulting from a cycle of continuous neutrophil recruitment and inactivation. This positive feedback loop that is outside of host control is one of the putative molecular mechanisms for excessive neutrophils, chronic inflammation, and collateral tissue damage associated with establishing a difficult to clear acute infection. Some have proposed the development of a hyper-inflammatory milieu to be indicative of initial establishment of a biofilm infection. Perhaps it is more precise to propose that establishment of a hyper-inflammatory milieu induces formation and adaptive evolution of a survival-enhanced protective biofilm. It may be best described as the window of opportunity in an otherwise healthy host that allow establish of a biofilm infection that is capable of persisting. Another important piece to the molecular puzzle of the formation of chronic biofilm infection is to determine how, once it is established, it maintains its niche in the patient—that is, what virulence factors produced by the biofilm enable it to persist in the patient.

EXPERIMENTAL MODEL OR MATERIAL: ADVANTAGES AND LIMITATIONS

Bianconi *et al.*⁸ developed a positive screening method called positive signature-tagged mutagenesis (Pos-STM) to create and select bacterial mutants that establish chronic airway infections in a mouse model. In this study, they found 16 mutants from an initial pool of 6,912 Pos-STM mutants after passage through four rounds of *in vivo* screening/selection that favors establishment of chronic infection. Only 12.5% of the mice that survived 14 days postinfection with wild-type *P. aeruginosa* developed chronic lung infection (Fig. 1). In contrast, 70%–100% of the surviving mice infected with the *P. aeruginosa* Pos-STM mutants developed chronic lung infections (Fig. 1). A limiting factor of this study is the time point used for recovering the positively selected mutants (14 days), which may not allow adequate time to select for

Figure 1. Virulence of *Pseudomonas aeruginosa* Pos-STM mutants in a murine model of airways infection.⁸ *P. aeruginosa* PAO1293 STM mini-Tn5 library that consisted of 6,912 mutants were sorted into 96 pools, each containing 72 mutants. Each pool was processed and injected into C57BL/6NCR1BR mice. The mice were sacrificed fourteen days post-infection and bacterial isolates were recovered by plating homogenates. Bacterial colony forming unit per lung and the percentage of infected mice were determined. In the first round of screening, bacterial pools that infected at least one of the two mice injected were selected and sorted into 21 pools containing 9–72 Pos-STM mutants per pool. Five mice were injected per pool. After the second round of screening, pools of *P. aeruginosa* mutants that infected more than 50% of the mice were selected and sorted into 15 pools containing 3–23 STM-mutants per pool. A total of 34 bacterial clones passed the third round of screening and were tested singularly in the fourth round using 10–15 mice per bacterial clone. *P. aeruginosa* STM mutants were identified by multiplex polymerase chain reaction. In the bottom graph fourteen days post-infection, mortality induced by bacteremia (black) and survival (light gray) was evaluated and reported as the percentage of mice infected. In the top graph fourteen days post infection, clearance (white) and capacity to establish chronic airways infection (dark gray) was determined on surviving mice and reported as the percentage of mice infected. The data represent two to three independent experiments (PAO1293 wildtype, n=10; STM mutants, n=10–15). Statistical significance by Chi square is indicated: *p<0.05, **p<0.01. Sixteen mutants that significantly increased chronic bAU3bAU4 infection (≥70%) when compared to PAO1293 (12.5%) were considered positive Pos-STM mutants and analyzed further.⁸ Pos-STM=positive signature-tagged mutagenesis.

many mutations that are required for establishing chronic biofilm infections. Phenotypic adaptations that were identified in previous studies of clinical isolates obtained late in chronic infection were not found, such as hypermutable mismatch-repair mutants or mucoid variants with increase expression of alginate EPS.⁸ These phenotypes may not be critical for establishment and/or early stages of chronic infection. Another limitation of this methodology is the number of initial Pos-STM mutants that are screened. This study theoretically covered only a fraction of the genes found in the *P. aeruginosa* genome.

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

The molecular mechanisms employed by bacteria to evade host immunity vary substantially and depend on the genetic makeup of each species. Studies have shown that bacteria in biofilms have increased rates of adaptive evolution under stress conditions that select for survival. The various mechanisms that promote genetic diversity that select for survival often produce heritable small-colony variants (SCVs).⁹ They were named SCVs because these *in situ* adapted mutant variants are

usually difficult to culture under standard laboratory conditions and produce small colonies on standard growth media. SCVs have been linked to the recurrence and persistence of chronic infections *in vivo*.^{10–12} For example, clinically isolated *P. aeruginosa* SCVs were demonstrated to correlate with enhanced fitness in CF airways to maintain chronic infections that evade/survive host immune response.¹³ These mutants likely represent biofilm virulence factor genes of chronic CF infection. As described above, several biofilm virulence factors have been identified using Pos-STM as loss-of-function mutations that enhance establishment and persistence of biofilm *in vivo*. Of the 15 genes identified, 4 of the Pos-STM mutants were related to motility, indicating that loss of motility favors establishment of chronic infection, which agrees with other studies.⁸ Interestingly, it has been shown that risk of bacteremia (acute planktonic infection) positively correlates with bacterial motility.¹³ They also showed two mutants with increased resistance to neutrophil killing favors establishment of chronic infection, which agrees with other studies that showed loss-of-function mutations in the same gene promotes persistence (biofilm formation).^{8,14} Five mutants showed significantly reduced stimulation of IL-8.⁸ Thus, altered stimulation of the immune response influences bacterial fitness to establish chronic infection (biofilms). A high number (six) of the Pos-STM mutants were related to metabolic functions, which supports previous studies that show that adaptations of bacterial metabolic pathways are key in developing chronic *P. aeruginosa* infections in CF patients.⁸ Sequence analysis of clinical isolates collected over a period of 16 years from seven CF patients with chronic infections identified loss-of-function mutants for 7 of the 15 Pos-STM genes identified in the mouse study.⁸ However, it is clear that bacteria in biofilms, particularly multispecies biofilms, also acquire new functions by various mechanisms, including genetic recombination, uptake of extracellular DNA, and direct horizontal gene transfer that help the bacteria establish chronic biofilms.¹⁴ A well-studied example is the acquisition of antibiotic resistance, a classic planktonic virulence factor, that has led to the emergence of bacteria resistant to new antibiotics. It is not difficult to imagine that many biofilm virulence

TAKE-HOME MESSAGE

Basic science advances

- A method was developed that identified bacterial mutants that had enhanced ability to generate chronic infections (biofilms) in a mouse lung infection model.
- This technique identified biofilm virulence factors genes that correlated with mutations in bacteria isolated from CF patients with chronic lung infections.
- Biofilm-adapted bacterial strains may derive from either loss-of-function or gain-of-function mutations.
- Planktonic bacteria favor pathogenic invasion/breaching molecular mechanisms that kill host cells.
- Bacteria in biofilms tend to employ defensive molecular mechanisms that maintain its niche in the patient and promote its survival resulting in chronic infection.

Clinical science advances

- Genetic assessments of clinical isolates from chronic infections have linked SCVs to the recurrence and persistence of chronic infections.
- Genetic analysis of clinical isolates identified biofilm virulence factors.

Relevance to clinical care

- Planktonic virulence factors underlie acute infection.
- Biofilm virulence factors underlie chronic infection.
- Bacteria often employ opposing molecular mechanisms to establish acute and chronic infections, thus requiring different strategies for treatment of acute and chronic (biofilm) infections.

factors that enhance establishment and persistence of biofilm infection may involve acquiring new functions rather than losing functions. An example of a gain-of-function alteration that has been identified in nontypeable *Haemophilus influenza* (NTHI) is the modification of the lipooligosaccharides (LOSs) of the bacteria's outer membrane that enhance biofilm formation and resistance to host innate immunity.⁶ Juneau *et al.*⁶ showed that expression of these modified LOSs in NTHI biofilm elicit the formation of NETs, thus bypassing neutrophil phagocytosis. NETs are host factors that consist of nuclear DNA fibers complexed with histones and granular proteins released through programmed cell death of neutrophils (NETosis) that function to entrap and kill bacteria.⁷ Studies have shown that NETs are unable to clear NTHI biofilm infection *in vitro* and that the presence of NETs structures containing viable NTHI biofilm positively correlate with high bacterial loads during otitis media infections.⁶ NTHI is a commensal of human nasopharynx, an opportunistic pathogen, and leading cause of chronic and recurrent otitis media biofilm infection.⁶

In summary, planktonic bacteria, particularly notoriously virulent species (*e.g.*, hyper-virulent community-associated methicillin-resistance *Staphylococcus aureus*), expressing well-studied classic planktonic virulence factors are historically associated with acute infection. In fact, planktonic bacteria are grouped as pathogens based on their synthesis of these virulence factors. (*e.g.*, cytolytic toxins like hemolysins that kill host cells, motility factors that enable bacteria to migrate rapidly through host tissues, proteases that break down host proteins that promote inflammation). Biofilms of both pathogenic and benign commensal species are now known to be associated with chronic infections and have been shown to evolve *in situ*, presumably generating biofilm infection-adapted variants, and express biofilm virulence factors.

INNOVATION

Bianconi *et al.*⁸ developed a methodology to select loss-of-function bacterial mutants that have enhanced ability to form chronic (biofilm) airway infections in a mouse lung infection model. In addition, they showed that these selected mutants are similar to clinical isolates found in CF patients with chronic airway infections (biofilms). This innovative methodology can be employed to rapidly screen broad comprehensive libraries of other bacterial species using lung or other chronic infection models to identify genes that enhance establishment and persistence of biofilm infections. The genes identified as biofilm virulence factors could then be included as targets in genotypic profile assessments during epidemiological studies to determine the combination of bacterial factors (chronic biofilm signature) that are potentially predictive of clinical outcomes resulting in different chronic biofilm infection pathologies.

CAUTION, CRITICAL REMARKS, AND RECOMMENDATIONS

One problem with the design of the Pos-STM study was the procedure Bianconi *et al.*⁸ used to determine biofilm formation. Biofilm formation was determined by relative absorbance of crystal violet staining of mutants each cultured in Luria-Bertani broth in 96-well plates for 18 h at 37°C. The optical density (OD)₆₀₀ of stained biofilm cultured

cells, normalized to the corresponding OD₆₀₀ of planktonic cultured cells, were assessed for each mutant. This procedure indicates the relative ability of each isolate to produce biomass within 18 h on standard media, which may not necessarily correlate with enhanced biofilm production *in vivo*. For example, SCV would not be expected to form significant biofilm mass under these conditions. This illustrates how the use of information that differentiates the molecular mechanisms employed by bacteria during acute and chronic infections can impact study design. A major limitation of the Pos-STM method, if used as developed in this study, is the bias toward loss-of-function mutations. Finally, the genes that enhance production of biofilms grown on an inert surface like polystyrene culture plates may not provide an adaptive advantage for forming biofilms on biological surfaces like dermal tissue.

FUTURE DEVELOPMENT OF INTEREST

Although many planktonic virulence factors are identified, only a few biofilm virulence factors are currently identified, and the mechanisms through which many of these genes function to promote biofilm formation are not well understood. Thus, much research remains to be done to identify and characterize additional biofilm virulence factors. Other than screening and characterization of clinical isolates combined with histological and epidemiological assessments, there are no comprehensive gain-of-function methods currently established for determining biofilm virulence factors. Identification of unique biofilm virulence factors could lead to the development of rapid point-of-care detectors that could assess the biofilm status and/or predict the healing course of wounds, and might provide new targets for antibiofilm therapies.

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