Pseudomonas aeruginosa forms Biofilms in Acute Infection Independent of Cell-to-Cell Signaling

J. Andy Schaber[1, 2], Jeff Triffo[3], Sang Jin Suh[4], Jeffrey W. Oliver[5], Mary Catherine Hastert[6], John A. Griswold[1], Manfred Auer[3], Abdul N. Hamood[2], Kendra P. Rumbaugh[1, 6*]

Departments of Surgery[1], Microbiology and Immunology [2] Pathology[5], and Cell Biology and Biochemistry[6] Texas Tech University Health Sciences Center, 3601 4th St., Lubbock, Texas, 79430, USA; Life Sciences Division, Lawrence Berkley National Laboratory, Berkley, California, 94720[3]; Department of Biological Sciences, Auburn University, Auburn, Alabama, 36849[4]

[*]Corresponding author:
Kendra Rumbaugh, Ph.D.
Texas Tech University Health Sciences Center
Department of Surgery
3601 4th Street
Lubbock, Texas 79430
phone: 806-743-2460, ext. 264
fax: 806-743-2370
e-mail: kendra.rumbaugh@ttuhsc.edu

Biofilms are bacterial communities residing within a polysaccharide matrix that are associated with persistence and antibiotic resistance in chronic infections. We show that the opportunistic pathogen *Pseudomonas aeruginosa* forms biofilms within 8 hours of infection in thermally-injured mice, demonstrating that biofilms contribute to bacterial colonization in acute infections. *P. aeruginosa* biofilms were visualized within burned tissue surrounding blood vessels and adipose cells. Although quorum sensing (QS), a bacterial signaling mechanism, coordinates differentiation of biofilms *in vitro*, wild type and QS-deficient *P. aeruginosa* formed similar biofilms *in vivo*. Our findings demonstrate that *P. aeruginosa* forms biofilms on specific host tissues independent of QS.

The opportunistic gram-negative bacterium *Pseudomonas aeruginosa* is one of the leading causes of morbidity and mortality in thermally injured patients (*1, 2*). *P. aeruginosa* pathogenesis in burn wounds has been extensively examined using the thermally-injured mouse model, which closely resembles human burn wound sequela (*3, 4*). In this mouse model, a low infecting dose (10^2 colony forming units (CFU)) of *P. aeruginosa* causes up to 100% mortality within 48 hours (*4*). Although bacterial biofilms have been associated with persistence and antibiotic resistance of *P. aeruginosa* in chronic infections, there is little information concerning the potential role of biofilm formation in acute infections, which are defined by short time courses and high severity. Therefore, in order to determine if biofilms form in acute burn wound infections, we used microscopic approaches to visualize bacterial infections *in situ* in mice administered full-thickness, third-degree scald burns and infected with a green fluorescent protein (GFP)-

expressing, wild-type strain of *P. aeruginosa*, PAO1 (*5*). We previously reported that PAO1 proliferates rapidly within the burn eschar, multiplying from a starting dose of 10^2 CFU to 10^9 CFU in less than 24 hours (*4*). Bacteremia is apparent in these mice as early as 24-hours post-burn/infection by the presence of *P. aeruginosa* in the blood, liver and spleen, and >90% of mice die within 48 hours post-burn/infection (*4*).

Tissues were harvested from the burn eschar at 8, 24 and 46 hours post-burn/infection. A third degree burn completely destroys the ultrastructure of the epidermis and dermis leaving only hypodermis, which is composed primarily of vascular, connective, muscle and adipose tissues. Thus, the burned epidermis and dermis were peeled away, homogenized and used to determine CFU (Fig. 1). Thin layers (approx. 1 mm) of the hypodermis were rinsed in sterile phosphate-buffered saline (PBS) and placed directly on slides for image analysis. Small clusters or microcolonies of GFP-expressing bacilli were visualized by confocal scanning laser microscopy (CSLM) at 8 hours post-burn/infection in all mice examined (n=6) (Fig. 2A). Microcolonies ranged in size from 14-33 μm. The CFU in these tissues had increased from the starting dose of 10^2 to $4.4x10^7 \pm 1.8x10^7$ (Fig. 1). Green fluorescence was not observed in burned but non-infected tissue samples, or burned tissue infected with non-GFP expressing PAO1 (data not shown). The CFU in the burned skin had increased to $1.3x10^9 \pm 3.9x10^8$ by 24 hours post-burn/infection and large bacterial aggregates or macrocolonies, ranging in size from 38-53 μm, were visualized in the tissues in 91% of the mice (10/11). These macrocolonies were primarily located surrounding adipocytes and veins (Figs. 2 and 3). All tissues harvested from PAO1-infected mice at 46 hour post-burn/infection exhibited extensive surface coverage (n=11), and aggregates measured 15-25 μm (Fig. 2).

Individual bacterial cells that were not associated with structures were also observed at all time points (Fig. 2).

Two distinctive clinical features of *P. aeruginosa* bacteremia are invasion and necrosis of blood vessels (*6*). Historically, blood vessel invasion by *P. aeruginosa* has been associated with the presence of bacilli in a circumferential pattern surrounding the vessel, where the bacterial cells are aligned single file or in stacks between cells of the venous walls (*6*). The formation of these structures is termed perivascular cuffing (PVC) (*7*). PVC was visualized in PAO1-infected tissues by CSLM , transmission electron microscopy (TEM) and fluorescence microscopy using a specific *P. aeruginosa* fluorescence *in situ* hybridization (FISH) probe (Fig 3C). PVC similar to that seen in *P. aeruginosa*-infected mouse tissues is commonly observed in human skin lesions termed ecthyma gangrenosum (*8*). Ecthyma gangrenosum is primarily associated with infections by the *Pseudomonas* and *Aeromonas* species and clinical diagnosis of *P. aeruginosa* infection is often based entirely on the recognition of these lesions (*7*). However, the mechanisms controlling the formation of PVC by *P. aeruginosa* and the role of PVC in pathogenesis are not fully understood. In this study, the detection of PVC in PAO1-infected tissue correlated strongly with the systemic spread of the bacteria to the liver and/or blood (n=14/15). Therefore, biofilm formation around blood vessels may be an important step leading to invasion of the vasculature and systemic spread of the bacteria.

Bacterial biofilms have been defined as groups of bacteria attached to a surface and enclosed in a matrix, typically made of polysaccharides, nucleic acids and proteins (*9*). Our CSLM images revealed large aggregates of *P. aeruginosa*, which were not removed by rinsing the tissue (Fig. 2 and 3). *P. aeruginosa* aggregates were visualized

by scanning electron microscopy (SEM) and TEM of the burned tissue to determine if they were associated with a biofilm matrix (BFM) (Fig. 2B, 4E and F and Supplementary Fig. 1). SEM images revealed matrix-like structures and/or 'bacterial flocs' in association with the *P. aeruginosa* aggregates (Fig. 2B). These structures are consistent with the polysaccharide biofilm matrices that have been described in *P. aeruginosa* biofilms *in vitro* (*10, 11*). For visualization by TEM, tissue sections were treated with ruthenium red, a polyanionic stain that stabilizes the structural integrity of the polysaccharide-rich BFM, which can be lost during the dehydration process (*12, 13*). Ruthenium red treated-tissue, counterstained with methylene blue revealed dark fiber-like structures between *P. aeruginosa* cells in TEM (Supplementary Fig. 1), which are consistent with previously demonstrated biofilms (*12, 13*). These fibrous structures were not visualized in areas devoid of *P. aeruginosa*.

The extracellular polysaccharide alginate is composed of mannuronic and guluronic acids and is a component of the *P. aeruginosa* BFM that may assist in protecting bacteria from antibiotics and host defenses in an infection (*14*). Alginate is produced by PAO1 *in vivo*, and alginate antibodies are detected in patients with extant *P. aeruginosa* infections (*15, 16*). We examined whether alginate was associated with *P. aeruginosa* vascular biofilms in thermally-injured mice. Deparafinized, PAO1-infected tissue sections were incubated with a monoclonal human anti-alginate antibody (*17*) and detected by fluorescence microscopy. A strong fluorescent signal was observed around blood vessels and adipocytes in samples from PAO1 infected tissues but not in non-infected tissues, tissues incubated with secondary antibody alone, or tissues treated with an irrelevant primary antibody (Fig. 4A, B and data not shown). To further confirm the

specificity of the alginate antibody, we performed immunohistochemical analysis on thermally injured mice infected with either an isogenic alginate mutant derived from PAO1 (PAO1 *algD1301*::*tet*), or a mutant strain complemented with a plasmid carrying the alginate synthesis genes (PAO1 *algD1301*::pALG2). Alginate signal was only detected in mice infected with the complemented mutant (compare Fig. 4C and D). To obtain higher resolution images, we utilized TEM to visualize tissues incubated with alginate primary antibodies and immunogold-labeled secondary antibodies. Gold particles were evident between individual bacterial cells *in vivo* (Fig. 4E and F), confirming that alginate is a component of the BFM surrounding bacteria *in vivo*. Taken together, these results indicate that *P. aeruginosa* rapidly forms aggregates that possess extracellular matrices in an *in vivo* acute infection model.

The differentiation or maturation of *P. aeruginosa* biofilms *in vitro* depends on intercellular signaling systems or QS (*18, 19*). QS systems in many gram-negative bacteria rely on acylated homoserine lactones (AHLs), which are produced at high levels when cell density is high and act as ligands for transcriptional regulators. The *P. aeruginosa* synthases LasI and RhlI synthesize two AHLs, N-3-oxododecanoyl homoserine lactone ($3OC_{12}$-HSL) and N-butyryl-homoserine lactone (C_4-HSL), which bind and activate the transcriptional regulators LasR and RhlR, respectively (*20*). These transcriptional regulators then initiate the transcription of many genes whose products, including proteases, elastases, toxins and hemolysins, are thought to be crucial for virulence (*20*). *P. aeruginosa* strains lacking functional QS systems are less virulent than wild type strains (*4*) and form flat, undifferentiated biofilms on glass surfaces (*18*). These undifferentiated biofilms are less stable than the differentiated biofilms formed by

wild type *P. aeruginosa* as they can be easily disrupted by the detergent sodium dodecyl sulfate (*18*). However, the role of QS in biofilm formation has not previously been examined *in vivo*.

In order to determine if a functional cell-to-cell signaling system is required for biofilm formation *in vivo*, we compared biofilm formation in thermally-injured mice infected with either PAO1 or an isogenic *P. aeruginosa* QS mutant strain (PAO1-JP2). PAO1-JP2 carries deletions in the *lasI* and *rhlI* genes, and does not synthesize $3OC_{12}$-HSL or C_4-HSL (*4*). PAO1-JP2 is also defective in twitching motility (*21*) and is significantly less virulent in the thermally-injured mouse model (*4*). Tissues from PAO1 and PAO1-JP2 infected mice were evaluated for bacterial load, presence of micro- or macrocolonies and PVC. Additionally, several features of PAO1 and PAO1-JP2 biofilms were quantitatively analyzed using COMSTAT (*22*), an image analysis program developed for analyzing structural elements in biofilms (Supplementary Table 1). CFU in the burn eschar were similar for both strains at 8, 24 and 46 hours, indicating that both can proliferate rapidly (Fig. 1). Morphological analyses revealed no major differences between the biofilms formed by PAO1 versus PAO1-JP2 (Fig. 2 and 3 and Supplementary Table 1). Specifically, PVC was visualized in 6/9 PAO1-infected mice and 4/9 PAO::JP2 at 24 hours post-burn/infection. Similarly, 5/6 PAO1-infected mice and 4/6 PAO1-JP2-infected mice displayed PVC at 46 hours post-burn/infection. In order to discount the possibility that the formation of PVC biofilms by PAO1-JP2 was due to reversion to wild type during passage in the mouse, we examined $3OC_{12}$-HSL synthesis in PAO1-JP2 colonies obtained from the liver and skin at 46 hours post-burn/infection utilizing the standard autoinducer bioassay (*23*). None of the PAO1-JP2

colonies examined produced $3OC_{12}$-HSL (data not shown). Analysis of COMSTAT data revealed no significant differences between any of the parameters studied, except that PAO1-JP2 displayed significantly less surface area coverage than PAO1 at 46 hour-post burn/infection (Supplementary Table 1). This supports our previous findings that PAO1-JP2 does not spread through the burn eschar as efficiently as PAO1 (*4*), and this phenotype is likely due to its defect in type IV fimbriae mediated twitching motility which facilitates bacterial translocation over moist surfaces (*24*). However, in most regards the *in vivo* biofilms made by PAO1-JP2 were similar to those made by PAO1. These data indicate that AHL-based cell-to-cell signaling is not required for rapid biofilm formation by *P. aeruginosa* within a burn wound.

We have previously determined that PAO1-JP2 causes less bacteremia and lower percent mortality than PAO1 (*4*), and these results were confirmed in this study (Fig. 1). However, the diminished systemic spread and decreased virulence of PAO1-JP2 was not due to its inability to form a biofilm. It is likely that the difference in virulence between PAO1 and PAO1-JP2 are due to defects in the expression of QS-regulated virulence factors in the mutant strain. It is possible that one or more of these factors are needed for efficient blood vessel invasion subsequent to biofilm formation. Using a PAO1 strain carrying a GFP reporter fused to the *rhlI* promoter, we detected GFP expression around blood vessels similar to that seen with the constitutive GFP reporter (Supplementary Fig. 2). This supports the contention that the role of biofilms in acute infections may be to achieve the high local cell density needed for expression of QS-controlled virulence factors crucial for systemic spread.

Figure Legend

Fig. 1. PAO1-JP2 causes less bacteremia and mortality in thermally-injured mice than PAO1. Female, Swiss Webster mice, weighing approx. 20 g were administered full-thickness, third degree scald burns as described previously (*20*). Mice were inoculated subcutaneously within the burn eschar with 10^2 CFU PAO1 (black bars) or PAO1-JP2 (white bars). The progression of the infection was assessed after 8, 24 and 46 hours by quantifying bacteria in the burned skin and liver and by observing mortality as described previously (*20*). The data for skin and liver colonization are expressed as mean $\pm$ s.e.m. (*P=0.04, **P=.001, Student's t-test). Mortality was determined at the 46 hour time point and was significantly decreased in PAO1-JP2 infected mice (P=0.002, Fisher's exact test).

Fig. 2. Biofilms are present around adipocytes in PAO1 and PAO1-JP2-infected tissue. Burned skin sections were harvested from mice infected with PAO1 or PAO1-JP2 after 8, 24 or 46 hours. The burned epidermis and dermis layers were removed and the underlying hypodermis (approx. 15x15x1 mm) was rinsed in sterile PBS. **(A)**, CSLM revealed micro or macro colonies of *P. aeruginosa* (bacteria appear white) predominately around adipocytes (labeled A). Rinsed skin sections were placed in imaging chambers containing an antifade reagent and imaged by CSLM. Skin sections were scanned (UPlan FL 20x/0.5) and z series were acquired at 1.0 μm intervals. Individual stack images were 3D reconstructed using MetaMorph 6.1 image analysis software. The scale bars (representing 65 μm) shown in the central plots are also valid for the right and lower

frames. **(B)**, Burned skin sections imaged by SEM at 7000x magnification revealed aggregates of *P. aeruginosa* adhered to adipocytes (labeled A), and coated with BFM or 'bacterial flocs' (labeled F).

Fig. 3. PAO1 and PAO1-JP2 form biofilms around veins. Burned skin sections from mice infected with PAO1 **(A)** or PAO1-JP2 **(B)** were harvested after 24 or 46 hours, respectively and longitudinal sections of *P. aeruginosa* PVC around veins were imaged by CSLM. Skin sections were scanned (UPlan FL 10x/0.3) and z series were acquired at 1.0 μm intervals (bacteria appear white). Shown in the right and lower frames are vertical sections through the biofilms collected at the positions indicated by the white triangles. The scale bars (representing 65 μm) shown in the central plots are also valid for the right and lower frames. The insets show bright field microscopy images of the tissue sections with veins clearly visible. **(C)** Cross section of a vein (UPlan FL 40x/1.30 oil) displaying *P. aeruginosa* PVC imaged by fluorescence microscopy. Bacteria were detected by a Cy3-labeled FISH probe and appear red around the vessel wall. Red blood cells within the lumen appear pink, and DAPI stained-host cell nuclei appear blue. **(D)** Cross section of a vein displaying *P. aeruginosa* PVC imaged by TEM (at 5,500x). Numerous bacilli (*Pa*B, *P. aeruginosa* biofilm) are visible surrounding the blood vessel wall (VW), red blood cells (RBC) are apparent in the vessel lumen.

Fig. 4. Alginate is present in the BFM surrounding *P. aeruginosa in vivo*. (**A-B)**, Immunohistochemical images of longitudinal sections of skin tissues from a thermally injured mouse infected with PAO1 after incubation with alginate primary monoclonal

antibody and Alexa fluor 488 secondary antibody (UPlan FL 40x/1.30 oil). Alginate is present in *P. aeruginosa* PVC **(A)** and surrounding adipocytes **(B)**. **(C-D)** Demonstration of alginate antibody specificity. Immunohistochemical analysis of blood vessels from thermally injured mice infected with PAO1 *algD1301*::*tet* **(C)** or PAO1 *algD1301*::pALG2 **(D)**. **(E-F)**, TEM micrographs of tissues from thermally injured mice infected with PAO1 **(E)** or PAO1-JP2 **(F)** incubated with alginate polyclonal antibody and an immunogold-labeled secondary antibody (scale bars represent 500 and 200 nm respectively). Immunogold particles are clearly located in the areas between cells, and are not associated with the cell membranes. These data therefore localize the alginate signal to the extracellular matrix, presumably in the BFM.

Supplementary Fig. 1. *P. aeruginosa* PVC in infected mouse tissue was viewed with ruthenium red staining and TEM. Cross section of a vein displaying *P. aeruginosa* PVC was imaged **(A),** 5500x, **(B),** 16,500x, **(C),** 68,750x and **(D),** 110,000x. Numerous bacilli (*Pa*B, *P. aeruginosa* biofilm) are visible surrounding the blood vessel wall (VW) , red blood cells (RBC) are apparent in the vessel lumen. *P. aeruginosa* extracellular matrix appears as dark fibers between cells as indicated by the arrow.

Supplementary Fig. 2. Quorum Sensing is induced in the aggregates surrounding veins. Burned skin sections from 3 mice infected with PAO1 carrying a *rhlI*::*gfp* promoter fusion were harvested after 24 hours and longitudinal sections of *P. aeruginosa* PVC around veins were imaged by CSLM as in Fig. 3. Shown is a representative image.

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