

1

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

3 **Cell Signaling**

4

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

7

6\*

8

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

14

15 \*Corresponding author:

16 Kendra Rumbaugh, Ph.D.

17 Texas Tech University Health Sciences Center

18 Department of Surgery

19 3601 4<sup>th</sup> Street

20 Lubbock, Texas 79430

21 phone: 806-743-2460, ext. 264

22 fax: 806-743-2370

23 e-mail: [kendra.rumbaugh@ttuhsc.edu](mailto:kendra.rumbaugh@ttuhsc.edu)

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

11  
12           The opportunistic gram-negative bacterium *Pseudomonas aeruginosa* is one of  
13 the leading causes of morbidity and mortality in thermally injured patients (1, 2). *P.*  
14 *aeruginosa* pathogenesis in burn wounds has been extensively examined using the  
15 thermally-injured mouse model, which closely resembles human burn wound sequela (3,  
16 4). In this mouse model, a low infecting dose ( $10^2$  colony forming units (CFU)) of *P.*  
17 *aeruginosa* causes up to 100% mortality within 48 hours (4). Although bacterial biofilms  
18 have been associated with persistence and antibiotic resistance of *P. aeruginosa* in  
19 chronic infections, there is little information concerning the potential role of biofilm  
20 formation in acute infections, which are defined by short time courses and high severity.  
21 Therefore, in order to determine if biofilms form in acute burn wound infections, we used  
22 microscopic approaches to visualize bacterial infections *in situ* in mice administered full-  
23 thickness, third-degree scald burns and infected with a green fluorescent protein (GFP)-

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

6 Tissues were harvested from the burn eschar at 8, 24 and 46 hours post-  
7 burn/infection. A third degree burn completely destroys the ultrastructure of the  
8 epidermis and dermis leaving only hypodermis, which is composed primarily of vascular,  
9 connective, muscle and adipose tissues. Thus, the burned epidermis and dermis were  
10 peeled away, homogenized and used to determine CFU (Fig. 1). Thin layers (approx. 1  
11 mm) of the hypodermis were rinsed in sterile phosphate-buffered saline (PBS) and placed  
12 directly on slides for image analysis. Small clusters or microcolonies of GFP-expressing  
13 bacilli were visualized by confocal scanning laser microscopy (CSLM) at 8 hours post-  
14 burn/infection in all mice examined (n=6) (Fig. 2A). Microcolonies ranged in size from  
15 14-33  $\mu\text{m}$ . The CFU in these tissues had increased from the starting dose of  $10^2$  to  
16  $4.4 \times 10^7 \pm 1.8 \times 10^7$  (Fig. 1). Green fluorescence was not observed in burned but non-  
17 infected tissue samples, or burned tissue infected with non-GFP expressing PAO1 (data  
18 not shown). The CFU in the burned skin had increased to  $1.3 \times 10^9 \pm 3.9 \times 10^8$  by 24 hours  
19 post-burn/infection and large bacterial aggregates or macrocolonies, ranging in size from  
20 38-53  $\mu\text{m}$ , were visualized in the tissues in 91% of the mice (10/11). These  
21 macrocolonies were primarily located surrounding adipocytes and veins (Figs. 2 and 3).  
22 All tissues harvested from PAO1-infected mice at 46 hour post-burn/infection exhibited  
23 extensive surface coverage (n=11), and aggregates measured 15-25  $\mu\text{m}$  (Fig. 2).

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

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

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

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

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

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

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

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

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

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

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

## 1 **Figure Legend**

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

12

13 **Fig. 2.** Biofilms are present around adipocytes in PAO1 and PAO1-JP2-infected tissue.  
14 Burned skin sections were harvested from mice infected with PAO1 or PAO1-JP2 after 8,  
15 24 or 46 hours. The burned epidermis and dermis layers were removed and the  
16 underlying hypodermis (approx. 15x15x1 mm) was rinsed in sterile PBS. (A), CSLM  
17 revealed micro or macro colonies of *P. aeruginosa* (bacteria appear white) predominately  
18 around adipocytes (labeled A). Rinsed skin sections were placed in imaging chambers  
19 containing an antifade reagent and imaged by CSLM. Skin sections were scanned (UPlan  
20 FL 20x/0.5) and z series were acquired at 1.0  $\mu$ m intervals. Individual stack images were  
21 3D reconstructed using MetaMorph 6.1 image analysis software. The scale bars  
22 (representing 65  $\mu$ m) shown in the central plots are also valid for the right and lower

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

4

5 **Fig. 3.** PAO1 and PAO1-JP2 form biofilms around veins. Burned skin sections from  
6 mice infected with PAO1 **(A)** or PAO1-JP2 **(B)** were harvested after 24 or 46 hours,  
7 respectively and longitudinal sections of *P. aeruginosa* PVC around veins were imaged  
8 by CSLM. Skin sections were scanned (UPlan FL 10x/0.3) and z series were acquired at  
9 1.0  $\mu\text{m}$  intervals (bacteria appear white). Shown in the right and lower frames are vertical  
10 sections through the biofilms collected at the positions indicated by the white triangles.  
11 The scale bars (representing 65  $\mu\text{m}$ ) shown in the central plots are also valid for the right  
12 and lower frames. The insets show bright field microscopy images of the tissue sections  
13 with veins clearly visible. **(C)** Cross section of a vein (UPlan FL 40x/1.30 oil) displaying  
14 *P. aeruginosa* PVC imaged by fluorescence microscopy. Bacteria were detected by a  
15 Cy3-labeled FISH probe and appear red around the vessel wall. Red blood cells within  
16 the lumen appear pink, and DAPI stained-host cell nuclei appear blue. **(D)** Cross section  
17 of a vein displaying *P. aeruginosa* PVC imaged by TEM (at 5,500x). Numerous bacilli  
18 (*PaB*, *P. aeruginosa* biofilm) are visible surrounding the blood vessel wall (VW), red  
19 blood cells (RBC) are apparent in the vessel lumen.

20

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

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

11

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

18

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

1 **Acknowledgments**

2 The authors would like to thank G.B. Pier for the generous gift of the alginate antibodies,  
3 S. C. Williams and M. R. Parsek for critical review of the manuscript, R. H. Veeh for  
4 critical assessment of biofilm images, M. Zemla for assistance in thin sectioning for  
5 immunolabeling, and B. Iglewski for PAO1-JP2 and pTdk-*rhlI-gfp*. This study was  
6 supported by the American Lung Association and South Plains Foundation (K.P.R.).

7

8

1 **References**

- 2 1. E. E. Tredget, H. A. Shankowsky, R. Rennie, R. E. Burrell, S. Logsetty, *Burns* 30,  
3 3 (Feb, 2004).
- 4 2. B. A. Pruitt, Jr., A. T. McManus, S. H. Kim, C. W. Goodwin, *World J Surg* 22,  
5 135 (Feb, 1998).
- 6 3. D. D. Stieritz, I. A. Holder, *J Infect Dis* 131, 688 (1975).
- 7 4. K. P. Rumbaugh, J. A. Griswold, B. H. Iglewski, A. N. Hamood, *Infect Immun*  
8 67, 5854 (Nov, 1999).
- 9 5. B. W. Holloway, V. Krishnapillai, A. F. Morgan, *Microbiol Rev* 43, 73 (1979).
- 10 6. R. Soave, H. W. Murray, M. M. Litrenta, *Am J Med* 65, 864 (Nov, 1978).
- 11 7. J. W. Oliver, Debowski, T. E., in *Check Sample*. (ASCP, Chicago, Ill, 1998).
- 12 8. D. M. Musher, *Hosp Pract (Off Ed)* 24, 71 (May 15, 1989).
- 13 9. J. W. Costerton, P. S. Stewart, E. P. Greenberg, *Science* 284, 1318 (1999).
- 14 10. M. Whiteley et al., *Nature* 413, 860 (Oct 25, 2001).
- 15 11. R. H. Veeh et al., *J Infect Dis* 188, 519 (Aug 15, 2003).
- 16 12. T. P. Fulcher et al., *Ophthalmology* 108, 1088 (Jun, 2001).
- 17 13. T. A. Fassel, C. E. Edmiston, Jr., *Methods Enzymol* 310, 194 (1999).
- 18 14. J. G. Leid et al., *J Immunol* 175, 7512 (Dec 1, 2005).
- 19 15. A. Bragonzi et al., *J Infect Dis* 192, 410 (Aug 1, 2005).
- 20 16. G. B. Pier, *Behring Inst Mitt*, 350 (Feb, 1997).
- 21 17. G. B. Pier et al., *J Immunol* 173, 5671 (Nov 1, 2004).
- 22 18. D. G. Davies et al., *Science* 280, 295 (1998).
- 23 19. M. R. Parsek, E. P. Greenberg, *Methods Enzymol* 310, 43 (1999).

- 1 20. K. P. Rumbaugh, J. A. Griswold, A. N. Hamood, *Microbes Infect* 2, 1721 (Nov,  
2 2000).
- 3 21. S. A. Beatson, C. B. Whitchurch, A. B. Semmler, J. S. Mattick, *J Bacteriol* 184,  
4 3598 (Jul, 2002).
- 5 22. A. Heydorn *et al.*, *Microbiology* 146 (Pt 10), 2395 (Oct, 2000).
- 6 23. J. P. Pearson *et al.*, *Proc Natl Acad Sci U S A* 91, 197 (1994).
- 7 24. J. S. Mattick, *Annu Rev Microbiol* 56, 289 (2002).

8

9