

REPORT DOCUMENTATION PAGE			Form Approved OMB NO. 0704-0188		
The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA, 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 08-04-2011		2. REPORT TYPE Final Report		3. DATES COVERED (From - To) 1-Sep-2006 - 28-Feb-2010	
4. TITLE AND SUBTITLE Final Report for Plant Biofilm Inhibitors to Discover Biofilm Genes			5a. CONTRACT NUMBER W911NF-06-1-0408		
			5b. GRANT NUMBER		
			5c. PROGRAM ELEMENT NUMBER 611102		
6. AUTHORS Thomas K. Wood			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAMES AND ADDRESSES Texas Engineering Experiment Station Research Services Texas Engineering Experiment Station (TEES) College Station, TX 77845 -4645			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Research Office P.O. Box 12211 Research Triangle Park, NC 27709-2211			10. SPONSOR/MONITOR'S ACRONYM(S) ARO		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S) 49621-LS.1		
12. DISTRIBUTION AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.					
14. ABSTRACT To control biofilms, we have synthesized the natural biofilm inhibitor (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone from the red alga <i>Delisea pulchra</i> and determined that it functions by disrupting quorum sensing in <i>Vibrio harveyi</i> by blocking all three channels of the <i>V. harveyi</i> quorum sensing system by rendering the quorum sensing master regulator protein LuxR unable to bind to the promoter sequences of quorum sensing-regulated genes. We have also discovered other inhibitors of cell signaling including indole, hydroxy					
15. SUBJECT TERMS biofilms, biofilm inhibitors					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Thomas Wood
a. REPORT UU	b. ABSTRACT UU	c. THIS PAGE UU			19b. TELEPHONE NUMBER 979-862-1588

Report Title

Final Report for Plant Biofilm Inhibitors to Discover Biofilm Genes

ABSTRACT

To control biofilms, we have synthesized the natural biofilm inhibitor (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone from the red alga *Delisea pulchra* and determined that it functions by disrupting quorum sensing in *Vibrio harveyi* by blocking all three channels of the *V. harveyi* quorum sensing system by rendering the quorum sensing master regulator protein LuxR unable to bind to the promoter sequences of quorum sensing-regulated genes. We have also discovered other inhibitors of cell signaling including indole, hydroxy indoles, and fluorouracil. We found indole is an interspecies signal that decreases *E. coli* biofilms through SdiA and increases the biofilms of pseudomonads. Importantly, we found that indole reduces the virulence of *P. aeruginosa* by decreasing quorum sensing phenotypes. In addition, we discovered uracil is a new second messenger in the cell which led to the discovery the fluorouracil is a potent anti-biofilm and anti-virulence compound. We also determined the role of prophage in the biofilm formation of *E. coli*, focused on toxin/antitoxins systems to show how they are related to persister cell formation and to show for the first time they are related to biofilm formation, and engineered some biofilm regulators so that biofilm formation may be controlled by rewiring cellular metabolism.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

1. "The Natural Furanone (5Z)-4-Bromo-5-(Bromomethylene)-3-Butyl-2(5H)-Furanone Disrupts Quorum Sensing-Regulated Gene Expression in *Vibrio Harveyi* by Decreasing the DNA-Binding Activity of the Transcriptional Regulator Protein LuxR," T. Defoirdt, C. M. Miyamoto, T. K. Wood, E. A. Meighen, P. Sorgeloos, W. Verstraete, and P. Bossier, *Environ. Microbiol.* 9: 2486-2495 (2007).
2. "YcfR (BhsA) Influences *Escherichia coli* Biofilm Formation Through Stress Response and Surface Hydrophobicity," X.-S. Zhang, R. Garcia Contreras, and T. K. Wood, *J. Bacteriol.* 189: 3051-3061, 2007.
3. "Indole is an Inter-Species Biofilm Signal Mediated by SdiA," J. Lee, A. Jayaraman, and T. K. Wood, *BMC Microbiology* 7:42, 2007. (highly accessed)
4. "Enterohemorrhagic *Escherichia coli* Biofilms are Inhibited by 7-Hydroxyindole and Stimulated by Isatin," J. Lee, T. Bansal, A. Jayaraman, W. E. Bentley, and T. K. Wood, *Appl. Environ. Microbiol.* 73: 4100-4109, 2007.
5. "R1 Conjugation Plasmid Increases *Escherichia coli* K-12 Biofilm Formation Through Envelope Stress Response (CpxAR and RseA), Biofilm Signaling (BssR), and OmpA," X. Yang, Q. Ma, and T. K. Wood, *Appl. Environ. Microbiol.*, 74:2690-2699, 2008.
6. "Indole Cell Signaling Occurs Primarily at Low Temperatures in *Escherichia coli*," J. Lee, X.-S. Zhang, M. Hegde, W. E. Bentley, A. Jayaraman, and T. K. Wood, *Nature ISMEJ* 2:1007-1023, 2008. (featured article)
7. "Potassium and Sodium Transporters of *Pseudomonas aeruginosa* Regulate Virulence to Barley," A. Ueda and T. K. Wood, *Appl. Microbiol. Biotechnol.* 79: 843-858, 2008.
8. "Protein Translation and Cell Death: The Role of Rare tRNAs in Biofilm Formation and in Activating Dormant Phage Killer Genes, R. Garcia-Contreras, X.-S. Zhang, and T. K. Wood, *PLoS ONE*, 3:2394, 2008.
9. "Uracil Influences Quorum Sensing and Biofilm Formation in *Pseudomonas aeruginosa* and Fluorouracil Is an Antagonist," A. Ueda, C. Attila, M. Whiteley, and T. K. Wood, *Microb. Biotechnol.*, 2:62-74, 2009. (featured article)
10. "Indole and 7-Hydroxyindole Diminish *Pseudomonas aeruginosa* Virulence," J. Lee, C. Attila, S. L. G. Cirillo, J. D. Cirillo, and T. K. Wood, *Microb. Biotechnol.* 2:75-90, 2009.
11. "Reconfiguring the Quorum-Sensing Regulator SdiA of *Escherichia coli* to Control Biofilm Formation via Indole and N-Acylhomoserine Lactones," J. Lee, T. Maeda, S. H. Hong, and T. K. Wood, *Appl. Environ. Microbiol.* 75: 1703-1716, 2009.
12. "Toxin-Antitoxin Systems in *Escherichia coli* Influence Biofilm Formation Through YjgK (TabA) and Fimbriae," Y. Kim, X. Wang, Q. Ma, X.-S. Zhang, and T. K. Wood, *J. Bacteriol.* 191: 1258-1267, 2009.
13. "5-Fluorouracil reduces biofilm formation in *Escherichia coli* K-12 through global regulator AriR as an antivirulence compound," C. Attila, A. Ueda, and T. K. Wood, *Appl. Microbiol. Biotechnol.*, 82: 525-533, 2009.
14. "Control and Benefits of CP4-57 Prophage Excision in *Escherichia coli* Biofilms," X. Wang, Y. Kim, and T. K. Wood, *Nature ISMEJ* 3:1164-1179, 2009. (featured article)
15. "Connecting Quorum Sensing, c-di-GMP, Pel Polysaccharide, and Biofilm Formation in *Pseudomonas aeruginosa* Through Tyrosine Phosphatase TpbA (PA3885)," A. Ueda and T. K. Wood, *PLoS Pathogens* 6:e1000483, 2009. (featured artwork).
16. "OmpA Influences *Escherichia coli* Biofilm Formation by Repressing Cellulose Production Through the CpxAR Two-Component System," Q. Ma and T. K. Wood, *Environ. Microbiol.* 11:2735-2746, 2009.
17. "Toxins Hha and CspD and small RNA regulator Hfq are involved in persister cell formation through MqsR in *Escherichia coli*," Y. Kim and T. K. Wood, *Biochem. Biophys. Res. Commun.* (2009) 391: 209-213, 2010.
18. "Three Dimensional Structure of the MqsR:MqsA Complex: A Novel Toxin:Antitoxin Pair Regulating Bacterial Persistence," B. L. Brown, S. Grigoriu, Y. Kim, J. M. Arruda, A. Davenport, T. K. Wood, W. Peti, and R. Page, *PLoS Pathogens* 5:e1000706, 2009.
19. "Escherichia coli Toxin/Antitoxin Pair MqsR/MqsA Regulate Toxin CspD," Y. Kim, X. Wang, X.-S. Zhang, W. Peti, and T. K. Wood,

Environ. Microbiol. 12: 1105-1121, 2010.

20. "Controlling biofilm formation, prophage excision and cell death by rewiring global regulator H-NS of Escherichia coli," Microbial Biotechnology 3: 344-356, 2010.

21. "Tyrosine phosphatase TpbA of Pseudomonas aeruginosa controls extracellular DNA via cyclic diguanylic acid concentrations," Environ. Microbiol. Reports 2: 449-455, 2010.

22. "Bacterial Quorum Sensing: Signals, Circuits, and Implications for Biofilms and Disease," A. Jayaraman and T. K. Wood, Annu. Rev. Biomed. Eng. 10: 145-167, 2008. (top 10 download)

23. "Insights on Escherichia coli Biofilm Formation and Inhibition from Whole-Transcriptome Profiling, T. K. Wood, Environ. Microbiol. 11:1-15, 2009.

Number of Papers published in peer-reviewed journals: 23.00

(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

Number of Papers published in non peer-reviewed journals: 0.00

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

(d) Manuscripts

Number of Manuscripts: 0.00

Patents Submitted

Patents Awarded

Awards

American Chemical Society Upstream Symposium Keynote Address (2008)

American Institute of Chemical Engineers Biochemical Engineering Plenary Award (Area 15C,2007)

Graduate Students

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
Can Attila	1.00
Qun Ma	0.50
Xiaole Yang	0.50
FTE Equivalent:	2.00
Total Number:	3

Names of Post Doctorates

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
Akihiro Ueda	1.00
Jintae Lee	0.25
Younghoon Kim	0.50
FTE Equivalent:	1.75
Total Number:	3

Names of Faculty Supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	National Academy Member
Thomas K. Wood	0.25	No
FTE Equivalent:	0.25	
Total Number:	1	

Names of Under Graduate students supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):.....	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

<u>NAME</u>

Total Number:

Names of personnel receiving PhDs

NAME

Can Attila

Xiaole Yang

Qun Ma

Total Number:

3

Names of other research staff

NAME

PERCENT SUPPORTED

FTE Equivalent:

Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

In the most recent year, we have primarily linked quorum sensing by AI-2 to the development of biofilm formation and persister cell formation (cells resistant to antibiotics that arise not through genetic change) through a novel toxin/antitoxin system MqsR/MqsA that we discovered as regulated during biofilm formation. To determine how this toxin MqsR functions, we isolated MqsR and MqsA and determined the X-ray crystallography structure of both the toxin and the antitoxin; this three-dimensional structure revealed MqsR is a novel RNase. In addition, we determined a new paradigm for the control of toxin/antitoxin modules: antitoxins may regulate not only their own synthesis but also control other toxin genes such as CspD (disrupts the membrane); this is the first example of a toxin/antitoxin pair regulating more than one loci. We also showed that deletion of mqsR results in reduced persister cell formation and this is only the second time a phenotype has been associated with a toxin gene. We are also the first lab to link toxin/antitoxin systems to biofilm formation.

In addition, in the past year we have linked for the first time a tyrosine phosphorylase (encoded by *tpbA*) to c-di-GMP and biofilm formation in *P. aeruginosa*. The *tpbA* mutant displays pleiotropic phenotypes such as hyper-biofilm formation, enhanced EPS production, altered colony morphology, increased aggregation, elevated c-di-GMP, and abolished swarming. Loss of an uncharacterized GGDEF protein, PA1120 (TpbB), suppressed these phenotypes, indicating that TpbA controls c-di-GMP production through TpbB. Therefore, the mechanism for QS-control of biofilm formation has been extended to include a novel phosphatase (TpbA), a diguanylate cyclase (TpbB), and c-di-GMP; hence, the predicted additional level of control of the *pel* polysaccharide locus has been identified and involves c-di-GMP as controlled by a tyrosine phosphatase.

In the past year we have also determined how OmpA controls biofilm formation in *E. coli*. OmpA influences biofilm formation differently on hydrophobic and hydrophilic surfaces since it represses cellulose production which is hydrophilic. A whole-transcriptome study revealed that OmpA induces the CpxRA two-component signal transduction pathway that responds to membrane stress and through this pathway cellulose production is affected. Cellulose is an important component in biofilm formation and virulence.

Over the course of the past year, we also determined how prophage influence biofilm formation. In *E. coli*, we found defective prophage are involved in host physiology via Hha and in biofilm formation by generating a diversified population with specialized functions in terms of motility and nutrient metabolism.

This year we also began to design engineered regulators to control biofilm formation based on our progress on understanding how biofilm forms at the genetic level. SdiA is a homolog of quorum-sensing regulators that detects N-acylhomoserine lactone (AHL) signals from other bacteria. *Escherichia coli* uses SdiA to reduce its biofilm formation in the presence of both AHLs and its own signal indole. We reconfigured SdiA (240 aa) to control biofilm formation using protein engineering by creating four SdiA variants including SdiA1E11 (F7L, F59L, Y70C, M94K, and K153X) and SdiA14C3 (W9R, P49T, N87T, frame shift at N96, and L139X) which reduced biofilm formation by 5- to 20-fold compared to wild-type SdiA in the presence of endogenous indole. Whole-transcriptome profiling revealed that wild-type SdiA reduced biofilm formation by repressing genes related to indole synthesis and curli synthesis, compared to no SdiA, while variant SdiA1E11 induced genes related to indole synthesis compared to wild-type SdiA. These results were confirmed by altered indole metabolism since variant SdiA1E11 produced 9-fold more indole which led to reduced swimming motility and cell density. Hence, wild-type SdiA decreased biofilm formation by reducing curli production and motility, and SdiA1E11 reduced biofilm formation further via indole. Furthermore, an AHL-sensitive variant (SdiA2D10 having four mutations at E31G, Y42F, R116H, and L165Q) increased biofilm formation 7-fold in the presence of N-octanoyl-DL-homoserine lactone and N-(3-oxododecatanoyl)-L-homoserine lactone. Therefore, SdiA can be evolved to increase or decrease biofilm formation, and biofilm formation may be controlled by altering sensors rather than signals.

Previously, to control biofilms, we have synthesized the natural biofilm inhibitor (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone from the red alga *Delisea pulchra* and determined that it functions by disrupting quorum sensing in *Vibrio harveyi* by blocking all three channels of the *V. harveyi* quorum sensing system by rendering the quorum sensing master regulator protein LuxR unable to bind to the promoter sequences of quorum sensing-regulated genes. We have also continued to study the effect of furanones on *Bacillus anthracis* with our collaborator Prof. Ranjan Srivastava at the University of CT. In addition, we utilized the furanones to protect nontoxic brine shrimp from pathogenic bacteria.

Beyond furanones, we have also discovered other inhibitors of cell signaling including indole/hydroxy indoles and 5-fluorouracil; these discoveries led to the initiation of two patent disclosures through Texas A & M University. We found indole is a quorum-sensing signal that decreases *E. coli* biofilms through SdiA and increases the biofilms of pseudomonads. We also showed that indole works primarily as a signal while *E. coli* is outside the human host and AI-2 works as a signal while *E. coli* is inside the human host. In addition, we found indole may be manipulated to control biofilm formation by oxygenases of bacteria that do not synthesize it in a dual-species biofilm. Furthermore, *E. coli* changes its biofilm in response to signals it cannot synthesize (homoserine lactones), and pseudomonads respond to signals they do not synthesize (indole). This promiscuous signal indole (made by *E. coli* but affecting *P. aeruginosa*) was found to decrease many of the *P. aeruginosa* virulence factors; hence, indole has potential for reducing the pathogenicity of this organism much like the way we found hydroxyindoles decrease biofilm and pathogenicity of enterohemorrhagic *Escherichia coli* O157:H7 (EHEC).

In both *E. coli* and *P. aeruginosa*, we simultaneously discovered that uracil, the building block of mRNA, serves as an intracellular second message that regulates quorum-sensing phenotypes. In *P. aeruginosa*, we found the importance of uracil by screening 5850 transposon mutants. If the cell could not make uridine monophosphate, then the quorum sensing phenotypes of elastase, pyocyanin, rhamnolipids, swarming and PQS were nearly completely abolished. This led to the discovery of 5-fluorouracil as a anti-virulence treatment against *P. aeruginosa* and *E. coli* that does not affect their growth (this compound is already approved for human use since it is used also to combat cancer). Hence, another new compound (like indole) was found that inhibits how bacteria communicate without affecting growth so there is likely to be resistance to it.

In addition, we also published the first report that linked toxin-antitoxin systems to biofilm formation by determining that the global regulator Hha of *E. coli* decreases biofilm formation by both repressing fimbriae formation and by inhibiting rare tRNA synthesis which leads to activation of dormant phage killer genes (prophage). Hence, we found the physiological importance of why the cell harbors cryptic prophage genes.

Technology Transfer