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CONTRACTING ORGANIZATION: North Carolina State University
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14. ABSTRACT

The Mid-Atlantic Microbial Pathogenesis Meeting was held Feb. 6-8, 2005 at the Wintergreen Ski Resort near Charlottesville, VA. Scientists working in all aspects of microbial pathogenesis attended the meeting and present their work. The meeting consisted of four sessions over two days and had 123 registered participants from 8 states. There were 25 speakers addressing various topics of microbial pathogenesis (4 invited speakers, 5 principal investigators, and 16 post-doctoral fellows and students). A poster session provided an additional opportunity for participants to present and discuss their work. Sixty-two posters were presented at this session.

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bacteria, pathogenesis, microbiology, virulence, disease

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Introduction

The Mid-Atlantic Microbial Pathogenesis Meeting was held Feb. 6-8, 2005 at the Wintergreen Ski Resort near Charlottesville, VA. The Mid-Atlantic Region comprised of North Carolina, Virginia, Maryland, The District of Columbia, Delaware, and New Jersey, is home to a large number of outstanding research groups that are engaged in the study of all aspects of microbial pathogenesis. These include researchers in academic, government and industrial laboratories. The 2005 meeting was the third annual Mid-Atlantic Microbial Pathogenesis Meeting. It was initiated in February of 2003 with funding aid from the USDA Animal Health and Well Being, the National Institute of Allergy and Infectious Diseases of the NIH, and the U.S. Army Medical Research and Materiel Command. The intent of the organizers was to maintain the meeting as an annual event based upon the success of the first two and the interest of the attendees in its continuation.

Body

Scientists working in all aspects of microbial pathogenesis attended the meeting and present their work. Topics of oral and poster presentations included the molecular basis of virulence, metabolism of microbial pathogens, host response, and vaccine development. Presentations included work both on bacterial pathogens, and on eukaryotic parasites.

The meeting consisted of four sessions over two days and had 123 registered participants from 8 states. Each session featured an invited keynote speaker and talks by principal investigators, post-doctoral fellows, and graduate and undergraduate students drawn from the submitted abstracts (a list of speakers and topics is provided below). In addition to the four keynote speakers, we had talks by 5 principal investigators and 16 post-doctoral fellows and students, for a total of 25 speakers. A poster session provided an additional opportunity for participants to present and discuss their work. Sixty-two posters were presented at this session. In addition, 54 travel awards were provided to selected post-doctoral fellows and graduate students who presented their work at the meeting, to defray the cost of registration and travel.

The meeting speakers, their affiliations, and their topics are listed here:

<u>Keynote Speakers:</u>

Michael Starnbach
Department of Microbiology and Molecular Genetics
School of Medicine at Harvard University
"Stimulating CD8+ T Cell Responses to Bacterial Pathogens"

Ferric C. Fang
Department of Microbiology and Laboratory Medicine
School of Medicine at University of Washington
"Nitric Oxide and the Respiratory Burst in Host Defense: Concepts and Controversies"

Vern Carruthers

Department of Molecular Microbiology and Immunology
Bloomberg School of Public Health
Johns Hopkins University
"*Toxoplasma* travel tips: For a quick journey to the center of a cell, pack lots of luggage and expect a tight squeeze"

Michele Swanson

Department of Microbiology and Immunology
School of Medicine at University of Michigan
"Pht is where it's at: amino acids signal *Legionella* to differentiate in macrophages"

Principal Investigators:

Stephen Melville – Virginia Tech
"Motility in a "non-motile" bacterium: Type 4 pili dependent movement of *Clostridium perfringens* resembles social motility in *Myxococcus xanthus"*

Tod J. Merkel – Food and Drug Administration
"Understanding the Early Steps in *B. anthracis* Infection of the Host"

Mary Hondalus – University of Georgia
"Pathogenesis of the Intracellular Actinomycete *Rhodococcus equi"*

Hongwei D. Yu – Marshall University
"Interleukin-12 is required for the innate host defense against lung colonization by *Pseudomonas aeruginosa"*

Rajendar Deora – Wake Forest University
"Regulation of biofilm development by the *Bordetella* BvgAS signal transduction system"

Post-doctoral Fellows and Graduate Students:

Nanette Fulcher – University of North Carolina at Chapel Hill
"Intracellular cAMP Control Mechanisms Regulate *Pseudomonas aeruginosa* Virulence Gene Expression"

Douglas R. Boettner – University of Virginia
"Identification of a putative transmembrane kinase with a role in phagocytosis of host cells by *Entamoeba histolytica"*

Matthew D. Mastropaolo – Virginia Tech
"Analysis of Bacterial Synergy in Polymicrobial Infections using a Type II Diabetic Mouse Model"

Susanne Bauman – Duke University

"Pseudomonas aeruginosa Outer Membrane Vesicles Export PaAP Aminopeptidase and Activate Human Lung Epithelia"

Lynette J. Crowther – University of Maryland
"Allosteric Stimulatory Effects of BfpC and BfpE on the Zn-ATPase BfpD of the Enteropathogenic *Escherichia coli* Bundle-Forming Pilus Biogenesis Machine"

Eric Anderson – East Carolina University
"Characterization of DhbR, A Transcriptional Activator Of Iron Acquisition Genes In *Brucella abortus"*

Belen Belete – Wake Forest University
"The Role Of AlgR And FimS In The Control Of Twitching Motility In *Pseudomonas Aeruginosa"*

Song-Ze Ding – University of Virginia
"Mitogen-Activated Protein Kinases Modulate Cell Cycle Progression in Gastric Epithelial Cells in the Absence or Presence of *Helicobacter pylori* Infection"

John V. McDowell - Virginia Commonwealth University
"Demonstration of the involvement of putative coiled-coil domains of the *Borrelia* Factor H binding proteins in interacting with human Factor H"

Antonio DiGiandomenico – University of Virginia
"Intranasal Vaccination Promotes Sterile Immunity to *Pseudomonas aeruginosa* Pneumonia"

Carol A. Gilchrist – University of Virginia
"Use of a virulence-associated gene microarray to analyze the changes in *E. histolytica* transcript levels during colonization of the mouse model of amebic colitis"

James T. Paulley – East Carolina University
"Transcription Of The *bhuA* Gene Of *B. abortus* Is Hemin Dependent And Required For Virulence In Experimentally Infected BALB/c Mice"

M. Sayeedur Rahman – University of Maryland
"Molecular and Functional Domain Analysis of *secA* Homolog from Rickettsiae"

Zoë E.V. Worthington – University of Maryland
"Investigating the Translocation of Pertussis Toxin from the ER to the Cytosol – Evidence for Exploitation of the ERAD Pathway"

Kelley M. Hovis – Virginia Commonwealth University
"Analysis of the Interaction of the *Borrelia hermsii* Factor H-Binding Protein, FhbA, and Factor H"

Jayasimha Rao – University of Virginia
"Identification of a Novel Protein (PA0122) from *Pseudomonas aeruginosa"*

Key Research Accomplishments

This meeting facilitated the exchange of research information regarding advances in the understanding of bacterial pathogenesis. It included researchers employed by both the government and academic institutions. Of particular interest to USDMRC, it included information on potential biowarfare and bioterrorism agents of military interest, including *Bacillus anthracis* and *Brucella* species.

Reportable Outcomes

The meeting generated a conference book that includes the abstracts of all of the oral and poster presentations. That book is included here as an appendix.

Conclusions

The goal of the organizers was to assemble a scientific program of sufficient quality to attract the region's many outstanding principle investigators along with their post-doctoral fellows and students. Many of the meeting's participants direct NIH- and/or USDA-sponsored research programs studying the pathogenesis of bacterial diseases. By providing a forum for these investigators to meet and discuss their work we hope to foster continuing collaborations between laboratories in the region and stimulate new ideas for scientific research. Another important goal of this meeting was to provide students and young investigators with a forum to describe their work. The active participation of both graduate students and post-doctoral fellows aids in the training of future scientists in the field of bacterial pathogenesis.

2005

Mid-Atlantic Microbial Pathogenesis Meeting

February 6-8, 2005

Wintergreen Resort
Wintergreen, VA

Meeting Sponsors

National Institute of Allergy and Infectious Disease
National Institutes of Health

Ensuring Food Safety Program
National Research Initiative
Cooperative State Research, Education and Extension Service
United States Department of Agriculture

United States Army
Medical Research and Materiel Command

Meeting Schedule and Organizers

Meeting Schedule

Sunday, February 6, 2005

2:00 - 4:30 **Meeting Registration and Check-in** – Wintergreen Resort Lobby – Coffee and light beverages

Session I
Moderator: Paul E. Orndorff, North Carolina State University

4:00 – 5:00 **Keynote Lecture**

Michael Starnbach
Department of Microbiology and Molecular Genetics
School of Medicine at Harvard University
"Stimulating CD8+ T Cell Responses to Bacterial Pathogens"

5:00 – 5:15 **Nanette Fulcher** – University of North Carolina at Chapel Hill
"Intracellular cAMP Control Mechanisms Regulate *Pseudomonas aeruginosa* Virulence Gene Expression"

5:15 – 5:30 **Douglas R. Boettner** – University of Virginia
"Identification of a putative transmembrane kinase with a role in phagocytosis of host cells by *Entamoeba histolytica"*

5:30 – 6:00 **Stephen Melville** – Virginia Tech
"Motility in a "non-motile" bacterium: Type 4 pili dependent movement of *Clostridium perfringens* resembles social motility in *Myxococcus xanthus"*

6:00 – 6:15 **Matthew D. Mastropaolo** – Virginia Tech
"Analysis of Bacterial Synergy in Polymicrobial Infections using a Type II Diabetic Mouse Model"

6:30 – 8:00 **Dinner** – The Devil's Grill

8:30 – 10:30 **Poster Session and Mixer**

Monday, February 7, 2005 - Morning

7:00 – 8:00 **Breakfast Buffet** – The Copper Mine
8:00 – 8:15 **Coffee** – outside meeting room

Session II
Moderator: C. Jeffrey Smith, East Carolina University

8:15 – 8:45 **Tod J. Merkel** – Food and Drug Administration
"Understanding the Early Steps in *B. anthracis* Infection of the Host"

8:45 – 9:00 **Susanne Bauman** – Duke University
"*Pseudomonas aeruginosa* Outer Membrane Vesicles Export PaAP Aminopeptidase and Activate Human Lung Epithelia"

9:00 – 9:15 **Lynette J. Crowther** – University of Maryland
"Allosteric Stimulatory Effects of BfpC and BfpE on the Zn-ATPase BfpD of the Enteropathogenic *Escherichia coli* Bundle-Forming Pilus Biogenesis Machine"

9:15 – 9:30 **Eric Anderson** – East Carolina University
"Characterization of DhbR, A Transcriptional Activator Of Iron Acquisition Genes In *Brucella abortus"*

9:30 – 9:45 **Belen Belete** – Wake Forest University
"The Role Of AlgR And FimS In The Control Of Twitching Motility In *Pseudomonas Aeruginosa"*

9:45 – 10:15 **Coffee break** – outside meeting room

10:15 – 11:15 **Keynote Lecture**

Ferric C. Fang
Department of Microbiology and Laboratory Medicine
School of Medicine at University of Washington
"Nitric Oxide and the Respiratory Burst in Host Defense: Concepts and Controversies"

11:15 – 11:30 **Song-Ze Ding** – University of Virginia
"Mitogen-Activated Protein Kinases Modulate Cell Cycle Progression in Gastric Epithelial Cells in the Absence or Presence of *Helicobacter pylori* Infection"

11:30 – 12:00 **Mary Hondalus** – University of Georgia
"Pathogenesis of the Intracellular Actinomycete *Rhodococcus equi"*

12:00 – 1:30 **Lunch buffet** – Copper Mine

Afternoon **Free time**

Monday, February 7, 2005 - Evening

5:00 – 6:45 **Dinner** – The Devil's Grill

Session III
Moderator: William Petri, University of Virginia

7:00 – 8:00 **Keynote Lecture**

Vern Carruthers
Department of Molecular Microbiology and Immunology
Bloomberg School of Public Health
Johns Hopkins University
"*Toxoplasma* travel tips: For a quick journey to the center of a cell, pack lots of luggage and expect a tight squeeze"

8:00 – 8:15 **John V. McDowell** - Virginia Commonwealth University
"Demonstration of the involvement of putative coiled-coil domains of the *Borrelia* Factor H binding proteins in interacting with human Factor H"

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"Transcription Of The *bhuA* Gene Of *B. abortus* Is Hemin Dependent And Required For Virulence In Experimentally Infected BALB/c Mice"

9:15 – 9:30 **M. Sayeedur Rahman** – University of Maryland
"Molecular and Functional Domain Analysis of *secA* Homolog from Rickettsiae"

Tuesday, February 8, 2005

7:00 – 8:15 **Breakfast Buffet** – The Copper Mine
8:15 – 8:30 **Coffee** – outside meeting room

Session IV
Moderator: Joanna B. Goldberg

8:30 – 9:00 **Hongwei D. Yu** – Marshall University
"Interleukin-12 is required for the innate host defense against lung colonization by *Pseudomonas aeruginosa"*

9:00 – 9:15 **Zoë E.V. Worthington** – University of Maryland
"Investigating the Translocation of Pertussis Toxin from the ER to the Cytosol – Evidence for Exploitation of the ERAD Pathway"

9:15 – 9:30 **Kelley M. Hovis** – Virginia Commonwealth University
"Analysis of the Interaction of the *Borrelia hermsii* Factor H-Binding Protein, FhbA, and Factor H"

9:30 – 9:45 **Jayasimha Rao** – University of Virginia
"Identification of a Novel Protein (PA0122) from *Pseudomonas aeruginosa"*

9:45 – 10:15 **Coffee break** – outside meeting room

10:15 – 11:15 **Keynote Lecture**

Michele Swanson
Department of Microbiology and Immunology
School of Medicine at University of Michigan
"Pht is where it's at: amino acids signal *Legionella* to differentiate in macrophages"

11:15 – 11:45 **Rajendar Deora** – Wake Forest University
"Regulation of biofilm development by the *Bordetella* BvgAS signal transduction system"

11:45 – 12:00 **Closing Remarks and Adjournment**

12:00 – 1:30 **Lunch buffet** – Copper Mine

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Oral Research Presentations

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Stimulating CD8+ T Cell Responses to Bacterial Pathogens

Michael Starnbach

Department of Microbiology and Molecular Genetics
School of Medicine at Harvard University

Research in my lab uses a combination of cellular and molecular approaches in the analysis of cellular immune responses to bacterial pathogens. Many virulence factors have been identified that allow bacteria to survive and replicate within the mammalian host and often within host cells. I have focused on the immune consequences of these survival strategies, specifically asking the following questions: What are the pathways for recognition of antigens from intracellular bacterial pathogens? How do bacterial virulence factors subvert or inhibit cellular immune responses? How can we promote the recognition of bacterial antigens that may or may not be recognized in the context of natural infections? What are the key host effector cells and pathways needed for protective immune responses to intracellular bacterial pathogens and their products?

A major component of my research program has centered on the immune recognition of *Chlamydia trachomatis.* This organism is the most common bacterial cause of sexually transmitted disease in the developed world and the leading cause of preventable blindness worldwide. We have shown that $CD8^+$ T-lymphocytes are stimulated during infection with *C. trachomatis* and we have been able to culture these T cells *in vitro*. These cultured cells have a protective effect when transferred into infected animals. The response of $CD8^+$ T cells is surprising as *C. trachomatis* is an obligate intracellular pathogen that is strictly confined to a vacuolar compartment. Using T cells, we are probing the interaction between *C. trachomatis* and the host cell. We have found that the antigens recognized by $CD8^+$ T cells are bound to the vacuolar membrane and have domains that protrude into the host cell cytosol. We are studying these cytosolic domains in an effort to understand what host factors interact with these antigens, and what role they may play in pathogenesis. In a parallel line of experimentation we are working to understand the requirements for processing *C. trachomatis* antigens and the effector functions of the T cells that respond. We have found that professional antigen presenting cells of hematopoietic origin are necessary for the initiation of a $CD8^+$ T cell response and that secretion of IFNγ by the T cells is required for protective function.

Other work in my lab has focused on the immune response to proteins secreted into host cells by bacteria of the genera *Salmonella*, *Shigella*, and *Yersinia*. During infection with these pathogens the normal response of T cells to these cytosolic antigens is inhibited. We are using a variety of genetic and biochemical approaches to define the mechanism of inhibition and the bacterial gene products responsible.

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Intracellular cAMP Control Mechanisms Regulate *Pseudomonas aeruginosa* Virulence Gene Expression

Presenting Author: Nanette Fulcher
Additional Authors: Erich Klem, Martin Cann, Matthew Wolfgang

Cystic Fibrosis/Pulmonary Research and Treatment Center, 7129 Thurston Bowles Bldg., CB #7248, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

Pseudomonas aeruginosa is an opportunistic human pathogen that can cause life-threatening lung infections in immunocompromised individuals and cystic fibrosis patients. *P. aeruginosa* possesses a wide variety of mechanisms implicated in virulence, including adherence and motility factors (Type IV pili and flagella), toxin expression and delivery systems (Type II and Type III secretion) and quorum sensing. These systems are tightly regulated and expressed in response to specific environmental cues. The transcription factor Vfr (Virulence factor regulator) controls the expression of over 200 genes, including those encoding components of multiple virulence mechanisms. Vfr activity is dependent on the small molecule second messenger adenosine 3', 5'-cyclic monophosphate (cAMP) and is a member of the cAMP receptor protein family. We have previously shown that mutants defective in the production of Vfr or cAMP are attenuated in a mouse model of acute infection, suggesting that regulation of cAMP synthesis may represent an important virulence factor control mechanism. Although the level of intracellular cAMP is elevated under specific conditions (e.g. calcium depletion or high salt), the mechanism by which cAMP levels are controlled in *P. aeruginosa* is poorly understood.

To further characterize the regulation of cAMP synthesis we focused on CyaB function as a possible control point. In *P. aeruginosa* cAMP is synthesized by either of two adenylate cyclase (AC) enzymes CyaA and CyaB. Under *in vitro* growth conditions CyaB is the major source of intracellular cAMP. CyaB has a modular structure consisting of two domains: i) a C-terminal ATP-binding catalytic domain, and ii) an N-terminal membrane-anchoring domain with six membrane-spanning segments termed Membrane-Associated Sensor 2 (MASE2). To study the enzymatic activity of CyaB we purified the predicted catalytic C-terminal domain under native conditions. Preliminary results indicate that this domain has enzymatic activity *in vitro* (~ 6.5 nmol cAMP/mg/min) and that this activity is severely inhibited by the bicarbonate ion. This finding is surprising given that the bicarbonate ion has been shown to directly stimulate cAMP production in related bacterial and mammalian ACs. Over-expression of the soluble C-terminal domain from a broad host range vector resulted in activation of cAMP-dependent genes in both *E. coli* and *P. aeruginosa*, suggesting that this domain is sufficient for catalytic activity. However, deletion of the N-terminal MASE2 domain from chromosomally-encoded *cyaB* resulted in reduced intracellular cAMP, indicating that the membrane-spanning domain plays an important role in enzyme function. We propose that extracellular signals such as those encountered during infection of the human host could be detected *via* the membrane-anchoring domain while intracellular signals such as metabolic status (bicarbonate) may act directly on the catalytic domain.

We also identified a gene encoding a homolog of the *E. coli* cAMP phosphodiesterase (CpdA), which degrades cAMP to AMP. A non-polar deletion of this gene in *P. aeruginosa* strain PAK resulted in altered expression of cAMP-dependent genes, increased basal levels of intracellular cAMP, and reduced growth rate. Overall, our results indicate that cAMP levels are highly regulated in *P. aeruginosa* and can be controlled at both the level of synthesis and degradation.

07

Identification of a putative transmembrane kinase with a role in phagocytosis of host cells by *Entamoeba histolytica*

Douglas R. Boettner[1], Christopher D. Huston[2], Ryan Petteway[3], and William A. Petri Jr.[3]
1. Microbiology, University of Virginia, Charlottesville, VA, USA
2. Medicine, University of Vermont, Burlington, VT, USA
3. Division of Infectious Diseases & International Health, University of Virginia, Charlottesville, VA, USA

Entamoeba histolytica is the causative agent of amebiasis, a disease associated with colitis and liver or brain abscesses. This infection is associated with massive tissue damage, which has been shown to involve the induction of apoptotic factors in host cells as well as the ingestion multiple cell types by the ameba. Since phagocytosis has been linked to virulence *in vivo*, the goals of this study were to identify and characterize possible participants in early phagocytosis events. In order to create a proteome of phagocytosis, we incubated ameba with carboxylated magnetic beads in intervals of time. Once beads were ingested, ameba were physically disrupted and intact phagosomes were isolated using a magnet. These phagosomes were delipidated and sequenced by liquid chromatography-mass spectrometry (LC-MS). The results of this analysis proposed the role for one transmembrane kinase (PhAK1) in phagocytosis. This was compelling given that there are around 100 transmembrane kinases present in the *Entamoeba histolytica* genome.

The intracellular portion of PhAK1 (containing the kinase domain) was expressed as a fusion with a 6X HIS tag in *Escherichia coli*. This protein was purified using a nickel agarose bead column and polyclonal serum was raised in NZW rabbits. Unfortunately by western blot this serum has shown to be broadly cross-reactive to between 9 and 11 other transmembrane kinases in *E. histolytica*. However, confocal microscopy using anti-PhAK1-kinase serum has shown that staining only occurred with permeablized cells indicating type 1 integral membrane proteins. This staining appeared as punctate surface staining. Following ingestion of human erythrocytes, the staining surrounded the phagosome. Although we have yet to indict PhAK1 in phagocytosis, these studies are suggestive, and will continue as we build more specific reagents. To date these are the first studies that have proposed a role for transmembrane kinases in the ingestion process of *E. histolytica*.

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Motility in a "non-motile" bacterium: Type 4 pili dependent movement of *Clostridium perfringens* resembles social motility in *Myxococcus xanthus*

Stephen Melville, John Varga, Van Nguyen, David O'Brien, Blair Therit, Richard Walker

Department of Biological Sciences, Virginia Tech, VA

Clostridium perfringens is a gram positive sporeforming bacterium that causes several human diseases, including gas gangrene and food poisoning. One of the defining characteristics of the species has been that it is non-motile. The complete genome sequences for 3 different strains of *C. perfringens* have recently been determined. The predicted ORFS reveal a complete absence of flagella and chemotaxis protein-encoding genes but genes encoding putative type IV pili proteins are present. We have demonstrated by video microscopy that *C. perfringens* exhibits motility at the leading edge of a colony grown on agar medium, reminiscent of the "flares" produced by the social motility of *M. xanthus*. The concentration of agar in the medium did not significantly affect the rate of movement. Electron microscopy revealed the presence of short filaments extending from the cell, and in some strains, a thick polysaccharide layer mediating cell-cell contact. The regulation of motility is at least partly dependent on the concentration of carbohydrates in the medium, where high levels of sugars such as glucose inhibit motility. This regulation may be mediated via the activity of the carbon catabolite regulatory protein CcpA, since a *ccpA*- strain was non-motile. We introduced mutations in genes encoding the putative PilC protein, thought to be involved in pili assembly, as well as PilT, which is involved in retraction of the type IV pili and movement of the cell. The *pilC*- and *pilT*- mutants were also non-motile. To determine if the synthesis of type IV pili was essential for *C. perfringens* gangrene infections, we injected high doses (108-109) of wild-type and mutant bacteria into the hind leg muscles of mice to initiate a gangrene infection. Interestingly, both the *pilT* and *pilC* mutant strains exhibited a lack of gangrene symptoms but the mice still died, although at a slower rate in the *pilC* mutant than the wild-type. In conclusion, we have shown for the first time to our knowledge that a gram positive bacterium exhibits twitching motility via a type IV pili and that this movement in *C. perfringens* is similar to social motility seen in *M. xanthus*.

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Analysis of Bacterial Synergy in Polymicrobial Infections using a Type II Diabetic Mouse Model.

Matthew D. Mastropaolo[1], Nicholas Evans[2], Meghan Byrnes[2], Ann M. Stevens[1], John Robertson[2], and Stephen B. Melville[1]

(1) Department of Biological Sciences and (2) Department of Biomedical Sciences and Pathobiology, Virginia Tech, Blacksburg, VA

Human diabetics frequently suffer delayed wound healing, increased susceptibility to localized and systemic infections, and limb amputations as a consequence of the disease. These infections are often polymicrobial in nature, involving mixtures of aerobic, facultative, and anaerobic bacteria. These bacteria frequently show synergy when inoculated together in animal models that survey abscess formation. Synergy is defined as a statistically significant increase in the number of bacteria in an infection with a second bacterium, as compared to that bacterium alone. The synergy has been hypothesized to depend on factors such as the ability to lower the oxygen concentration of host tissues, synthesis of a capsule, and production of cytotoxins. The purpose of this study is to determine if *Escherichia coli*, *Bacteroides fragilis*, and *Clostridium perfringens* contribute to synergy in polymicrobial infections using type II diabetic mice as an in vivo model. The strain of mice we used for these experiments is BKS.Cg-*m* +/+ *Lepr*^{db}. Two populations of mice were compared: young (5-6 weeks old) prediabetic mice and aged (23-24 weeks old) mice that exhibit hyperglycemia by 4-6 weeks of age. Mice of strain C57BLK/J of the same age were used as non-diabetic controls. Our dual hypothesis is that each bacterial species will be synergistic in the polymicrobial infections and that diabetes and not age is a factor in susceptibility to polymicrobial infections. The mice were injected subcutaneously in the inner thigh with single and mixed cultures containing 1 x 10^6 each of *E. coli*, *B. fragilis*, and *C. perfringens* in all possible combinations. Progression of the infection was monitored by examining bacteriological and immunological indicators at 1, 8, and 22 days post-infection. Abscess formation was monitored and the area of abscess formation was examined for pathological changes during the course of the infection. Synergy was observed between *E. coli* and *B. fragilis* in both the young group and aged group of mice. The aged mice carried a higher bacterial burden than the young mice. No difference in bacterial counts was seen in the control strain of mice, giving support to our hypothesis that age is not a factor in the susceptibility to polymicrobial infections. Work is underway to develop chromosomal mutants of two key virulence factors and the three terminal oxidases of the *E. coli* strain to determine if they play a role in synergy.

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Understanding the Early Steps in *B. anthracis* Infection of the Host

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Bacillus anthracis, the etiological agent of anthrax, is a Gram-positive, aerobic, spore-forming bacterium. Dormant spores are highly resistant to adverse environmental conditions, and are able to re-establish vegetative growth in the presence of favorable environmental conditions. Anthrax is initiated by the entry of spores into the host through the skin, via the gastrointestinal tract, or through the respiratory epithelium after inhalation of airborne spores. The inhalation form of anthrax is the most severe and is associated with rapid progression of the disease and death. The vegetative form of *B. anthracis* produces a number of virulence factors including specific toxins. The best described virulence determinants of *B. anthracis* are encoded on two large virulence plasmids (pXO1; 185 kb and pXO2; 97 kb. The three genes that encode the proteins that combine to form the *B. anthracis* toxins (*cya lef* and *pag*) are found on the pXO1 plasmid. The combination of protective antigen and edema factor (edema toxin) causes edema when injected subcutaneously, and the combination of protective antigen and lethal factor (lethal toxin) causes death when injected intravenously. A capsule composed of poly-D-glutamic acid is encoded on the pXO2 plasmid. The capsule is believed to protect vegetative cells from microbicidal activity and serum proteins. Very little is known about the host immune response to *B. anthracis* infection. Systematic examination of the role of the various components of the immune response in controlling anthrax after aerosol exposure has not been performed. A more complete understanding of the host immune response and specifically, the identification of those elements of the immune response that contribute to control and clearance of *B. anthracis* infection following an aerosol exposure will make significant contributions towards our understanding of the host response to *B. anthracis* infection and towards the development of new vaccines and post-exposure treatments. In order to begin to address these questions, we have undertaken anthrax challenge experiments using mice with specific genetic defects (knock-out mice) that eliminate defined components of the immune response, and wild-type mice that have been depleted of specific immune effector cell types. We have examined the contribution of the complement system, alveolar macrophages and neutrophils to the control of *B. anthracis* aerosol infection using the mouse aerosol challenge model. We have found that complement factor 5 (C5) plays a crucial role in the containment of *B. anthracis* and that normally resistant mice can be made susceptible to the *B. anthracis* Sterne strain by pre-treatment with the anti-complement factor, cobra venom factor (CVF). We have determined that alveolar macrophages contribute minimally to protection from aerosolized *B. anthracis* spores. In contrast, neutrophils play a critically important role in the containment of infection following aerosol exposure.

05

Pseudomonas aeruginosa Outer Membrane Vesicles Export PaAP Aminopeptidase and Activate Human Lung Epithelia

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Pseudomonas aeruginosa is a gram negative, opportunistic pathogen that is a major cause for morbidity and mortality in individuals with compromised lung function such as in patients with cystic fibrosis (CF). One major cause of lung injury results from the acute inflammatory response to the infection. Vesicles, consisting of periplasmic and outer membrane proteins and lipids, are secreted by *P. aeruginosa* as well as many other well-characterized pathogens. We characterized vesicles produced by *P. aeruginosa* and investigated their interactions with human respiratory cells and the consequent immune response. Vesicles from CF strains exhibited the highest association with human lung epithelia. Sequencing of vesicle proteins revealed that the aminopeptidase PaAP (PA2939) was enriched in vesicles from CF strains compared to vesicles from other strains. Furthermore, full-length PaAP was enriched in vesicles compared to periplasmic and outer membrane fractions and PaAP appeared in the supernatant predominantly in a high molecular weight complex (>100 kDa), suggesting it is preferentially exported via vesicles. Vesicle-associated PaAP in vesicles was found to be active and exteriorly located. Since vesicles are likely to come into contact with host cells during an infection, we investigated the cellular response to vesicles from *P. aeruginosa* strains of different origins. The induction of IL-8 by vesicles was significantly higher than that elicited by a 10-fold higher amount of purified LPS from the same strain, suggesting that LPS alone was not responsible for the IL-8 response. These results suggest that *P. aeruginosa* infecting the CF lung produce vesicles enriched in a specific protease that associate with lung cells and can contribute to the inflammatory response.

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Allosteric Stimulatory Effects of BfpC and BfpE on the Zn-ATPase BfpD of the Enteropathogenic *Escherichia coli* Bundle-Forming Pilus Biogenesis Machine

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Although type IV pilus biogenesis, protein secretion, DNA transfer and filamentous phage morphogenesis systems are involved in diverse processes, they are thought to possess similar architectures and mechanisms. These multiprotein complexes include members of the PulE superfamily of putative NTPases that have extensive sequence similarity and probably similar functions as the energizers of macromolecular transport. We have purified the PulE homologue BfpD of the bundle-forming pilus (BFP) biogenesis machine of enteropathogenic *Escherichia coli* and characterized its ATPase activity, providing new insights into its mode of action. Gel filtration and electron microscopy revealed that BfpD forms hexamers in the presence of nucleotide. Hexameric BfpD displayed weak ATPase activity that was highest in the presence of Zn^{2+}. We previously demonstrated that BfpD binds to the cytoplasmic N termini of cytoplasmic membrane proteins BfpC and BfpE. Here, we identified two BfpD-binding sites, $BfpE_{39-76}$ and $BfpE_{77-114}$, in the N terminus of BfpE using a yeast two-hybrid system. Isothermal titration calorimetry and protease sensitivity assays showed that hexameric BfpD-ATPγS binds to $BfpE_{77-114}$, while hexameric BfpD-ADP binds to $BfpE_{39-76}$. Interestingly, the N terminus of BfpC and $BfpE_{77-114}$ together increased the ATPase activity of hexameric BfpD over 1200-fold to a V_{max} of 75.3 μmol P_i min^{-1} mg^{-1}. We conclude that BfpC and BfpE recruit hexameric BfpD to the BFP biogenesis machine and dramatically stimulate its ATPase activity. Furthermore, we propose a model for the mechanism by which BfpD transduces mechanical energy to the BFP biogenesis machine, involving differential nucleotide-dependent binding of hexameric BfpD to $BfpE_{39-76}$ and $BfpE_{77-114}$.

02

Characterization of DhbR, A Transcriptional Activator Of Iron Acquisition Genes In *Brucella abortus*

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Brucellosis is a zoonotic disease caused by the Gram–negative intracellular pathogens comprising the bacterial genus *Brucella*. In the natural ruminant host, brucellosis leads to reproductive failure, manifesting as either abortion, or the birth of severely weakened offspring. In humans, brucellosis results in a long-term, cyclic, flu-like illness known as undulant or Malta fever.

Iron is essential to the survival of *Brucella abortus*, but the mammalian host represents an extremely iron-restricted environment. In response to this restriction, *Brucella* synthesizes two catechol-type siderophores, 2, 3-dihydroxybenzoic acid (DHBA) and the more complex siderophore, brucebactin. Both are produced through the enzymatic activities of the products of the *dhb* operon, and expression of this operon is tightly regulated in response to environmental iron levels. Two consensus Fur boxes are located within the *dhb* promoter, suggesting regulation by Fur, but an isogenic *fur* mutant constructed from *B. abortus* 2308 displays wild-type repression of *dhb* expression in response to iron-replete growth conditions, indicating that an alternate regulator controls expression of the *dhb* operon.

One strategy employed by some bacteria to regulate siderophore biosynthesis is the use of AraC-like transcriptional activators. Examples of these activator proteins are YbtA (yersiniabactin A) of *Yersinia pestis* and AlcR (alcaligin biosynthesis regulator) of *Bordetella bronchiseptica*. In these organisms, the end product siderophore serves as a co-activator in conjunction with the AraC-like protein, and this activation is iron-responsive.

Brucella abortus 2308 possesses a homolog of the *B. bronchiseptica* AlcR. An isogenic *B. abortus alcR* mutant, BEA5, shows decreased catechol siderophore production under iron-deplete conditions, when compared to the parental 2308 strain, suggesting that the product of this gene, termed DhbR (dihydroxybenzoic acid regulator), functions as an activator of siderophore biosynthesis. Additional studies indicate that this regulation occurs at the level of transcription and is the result of direct interactions between DhbR and the *dhb* promoter region.

While DhbR is required to achieve maximal siderophore production, experimental evidence suggests the presence of an additional level of iron-responsive regulation for the *dhb* operon. The nature of the complex interplay between DhbR and other potential iron-responsive transcriptional regulators in controlling expression of the *dhb* operon is currently under investigation.

06

The Role Of AlgR And FimS In The Control Of Twitching Motility In *Pseudomonas Aeruginosa*.

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Pseudomonas aeruginosa is an opportunistic pathogen that can infect immunocompromised individuals and cystic fibrosis patients. This bacterium produces a wide range of virulence factors including the exopolysaccharide alginate and type IV fimbriae. Type IV fimbriae retraction and extension mediate twitching motility, which is a flagella-independent mode of solid surface translocation. Both twitching motility and pilus production are essential for *P. aeruginosa* virulence. The FimS/AlgR sensor-regulator pair regulates the biosynthesis of type IV fimbriae (pili). At present, the mechanism by which AlgR controls twitching motility is unknown. Most response regulators require phosphorylation at an aspartate residue for their activity. AlgR is phosphorylated at Asp54 and mutating this residue results in the loss of twitching motility. Deletions of both *algR* and *fimS* also result in the loss of twitching motility. Western blot analyses on whole cell lysates of *algR* mutants show no defects in pili production. However, transmission electron microscopy and Western blot analyses on surface sheared samples reveal that *algR* mutants lack surface expressed pili. Cellular fractionation studies on the *algR* mutants show that pilin monomers are trapped in the cytoplasmic space and fail to reach the periplasmic space. Thus, while *algR* mutations do not affect pili production, it is clear that AlgR plays an important role in modulating proper pili localization. Data from adherence assays also show that both the *algR* deletion mutant and the *algR* phosphorylation mutant are significantly reduced in their ability to adhere to human bronchial epithelial cells. These findings indicate that *algR* is required for both twitching motility and adherence. Thus, the overall effects of mutating *algR* suggest a decrease in *P. aeruginosa* virulence.

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Nitric Oxide and the Respiratory Burst in Host Defense: Concepts and Controversies

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Phagocyte-derived reactive oxygen and nitrogen species (ROS/RNS) produced by the NADPH phagocyte oxidase and inducible nitric oxide synthase, respectively, are of crucial importance for host resistance to microbial pathogens. The versatility of ROS/RNS as signaling and cytotoxic molecules allows these molecules to have multiple roles in infection through a variety of mechanisms. Their ability to target multiple essential processes of microbial pathogens and to synergize with each other, or with oxygen-independent antimicrobial systems, results in enormously broad antimicrobial activity that is difficult for microorganisms to completely resist or circumvent. Decades of research have provided a detailed understanding of the regulation, generation and actions of these molecular mediators together with their roles in resisting infection. Analysis of genetically determined variability in ROS and RNS production by humans or laboratory mice has helped to clarify the role of ROS and RNS in host defense. However, differences of opinion remain with regard to their host specificity, cell biology, sources, and interactions with each other or with myeloperoxidase and granule proteases. More than a century after Metchnikoff described phagocytosis and more than four decades after the discovery of the burst of oxygen consumption associated with microbial killing, the seemingly elementary question of how phagocytes inhibit, kill and degrade microorganisms remains controversial, ensuring that biologists interested in the interactions between host and microorganisms still have many fascinating questions to answer.

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Mitogen-Activated Protein Kinases Modulate Cell Cycle Progression in Gastric Epithelial Cells in the Absence or Presence of *Helicobacter pylori* Infection

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Helicobacter pylori infection is associated with alterations of epithelial cell growth and differentiation. It also induces up-regulation of mitogen-activated protein kinases (MAPK) including ERK, p38, and JNK. In other systems, MAPK have been shown to play an important role in mediating cell cycle, proliferation, and differentiation. However, it has not been established whether MAPK modulate gastric epithelial cell cycle in response to *H. pylori* infection. The goal of the current study was to determine if MAPK members differentially regulate cell cycle in gastric epithelial cells in the absence or presence of *H. pylori* infection.

In the absence of *H. pylori* infection, the JNK inhibitor (SP600125) increased ERK and p38 activation in gastric adenocarcinoma cell line, AGS; the p38 inhibitor (SB202190) only increased ERK activation; and ERK inhibitor (PD98059) had no effect on either p38 or JNK activation. Both ERK and JNK inhibitors increased p21 and p27, and had no effect on cyclins A, B. Only JNK inhibitor increased cyclin E and D1 expression. The p38 inhibitor also increased cyclin E, decreased p21 and p27 and had no effect on cyclin A and B and D1 expression. The final results of these changes in the expression of cell cycle machinery are reflected in the modulation of the cell cycle. ERK inhibitor arrested AGS cell cycle at G_1 phase; p38 inhibitor promoted the progression of the cell cycle to the S phase; and JNK inhibitor promoted the progression of the cell cycle to the G_2 phase.

Infection of *H. pylori* (MOI=300:1) resulted in consistent ERK, p38, and JNK activation in AGS cells. *H. pylori* arrested the cell cycle at G_1 phase over 24 hours with an increase in p21, p27, cyclin E and a decrease in cyclins A, B, and D1 expression. In the presence of *H. pylori* infection, JNK inhibitor had little effect on ERK or p38 activation; the p38 inhibitor slightly increased JNK and ERK activation; and ERK inhibitor decreased JNK activation, but not p38 activation. The ERK and p38 inhibitors did not have a consistent or significant effect the *H. pylori* induction of any of the above cell cycle components. At an MOI=150:1, the JNK inhibitor partially restored the levels of cyclin A, B, and D1 expression, and abolished the *H. pylori*-induced cell cycle inhibition, although it had little effect on p21, p27 and cyclin E.

Our data demonstrate that *H. pylori* induced consistent ERK, p38 or JNK activation in gastric epithelial cells. The MAPK members affect one another and coordinately regulate the expression of cell cycle machinery and modulate cell cycle progression. The final outcome of *H. pylori* infection likely depends upon the interaction between bacteria and these MAPK networks.

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Pathogenesis of the Intracellular Actinomycete *Rhodococcus equi*

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With the arrival of the HIV epidemic, and the increase in individuals undergoing chemotherapy for various cancers, the understudied, zoonotic opportunistic pathogen *Rhodococcus equi*, has emerged as cause of a life-threatening granulomatous pneumonia in persons of compromised immunity. *R. equi* is a common soil actinomycete, which when inhaled by susceptible individuals, resists innate killing mechanisms and readily multiplies in unactivated macrophages. Like other members of the Actinomycete genera, intramacrophage replication of *R. equ*i is associated with the disruption of normal phagosome processing events. While *R. equi* is a relatively newly identified opportunistic pathogen of immunocompromised humans, it has long been recognized as one of the most important veterinary bacterial pathogens causing a similar pulmonary disease in young neonatal horses (foals). Nonetheless, little is known about the molecular basis for *R. equi* pathogenesis largely because prior to our recent work, few tools existed to allow a thorough genetic analysis of this bacterium. We recently developed the genetic methods to construct defined mutants of *R. equi*. Using these new tools, we demonstrated that *vapA* (virulence associated protein A) a gene present on the virulence plasmid of *R. equi*, is required for full virulence. *vapA* is a member of a novel, *Rhodococcus* spp. Specific, gene family which lacks homologs in other bacterial species. *vapA* encodes a surface-expressed lipoprotein of unknown function that is required for macrophage intracellular replication. We believe that *vapA* plays a crucial role in altering the intracellular environment, thereby promoting the survival and replication of *R. equi* in phagocytic cells. We have found that recombinant VapA protein (rVapA) added *in trans* (in solution) to infected macrophage monolayers, will rescue the attenuated phenotype of the *vapA* deletion mutant, allowing it to replicate within macrophages at wild type levels. This effect is VapA specific, as the similar addition of irrelevant proteins has no effect. In addition, our data indicate that macrophage exposure to rVapA affects the efficiency of the phagosome development process. These observations are consistent with the hypothesis that VapA influences post-phagocytic events. A focus of our ongoing research is to define the role of *vapA*, and in so doing, we may uncover a unique mechanism utilized by an intracellular opportunistic pathogen to enable survival in the host.

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Toxoplasma travel tips: For a quick journey to the center of a cell, pack lots of luggage and expect a tight squeeze

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Intracellular pathogens have evolved a spectrum of strategies for invading cells during infection. At one end of this scale, entry by many pathogens is relatively passive, relying mainly on host internalization by phagocytosis or endocytosis. Other pathogens including several gram negative bacteria enter semi-actively by inducing host cytoskeletal rearrangements and engulfment. At the other end of the spectrum, fully-active invasion by a group of intracellular apicomplexan parasites including *Toxoplasma gondii* proceeds through elaborate series of events critically dependent on substrate dependent motility and protein secretion. While initial, reversible binding to the target cell is likely mediated by surface antigens (SAGs), irreversible attachment is achieved by the mobilization of adhesins from anterior secretory granules called micronemes (MICs) to form a surface adhesion zone, the moving junction. After the release of other invasion organelles called rhoptries (ROPs), *Toxoplasma* penetrates into the target cell using its intrapellicular motor system to posteriorly translocate the moving junction, which is visible as a prominent constriction. Completed in 30 seconds or less, this invasion strategy appears to be highly coordinated, efficient, and effective for entering a wide assortment of host cells. New insight into the timing, regulation, and parasite products involved in *Toxoplasma* entry will be discussed along with emerging strategies to interfere with parasite invasion.

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Demonstration of the involvement of putative coiled-coil domains of the *Borrelia* Factor H binding proteins in interacting with human Factor H

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Lyme disease (LD) is the most common tick-borne zoonosis in the United States with approximately 24,000 cases reported annually to the CDC. LD is caused by the spirochete *Borrelia burgdorferi*, which utilizes multiple mechanisms for immune evasion and is capable of establishing a chronic infection in the absence of treatment. It has recently been demonstrated that these bacteria express two classes of proteins which can bind human factor H (fH); these include the OspE paralogs and BBA68. fH is the second most highly abundant serum protein and is an important regulator of the alternative complement cascade. Several human pathogens have been shown to bind fH to their surface, a process that facilitates immune evasion or cell to cell interaction. The molecular basis of the interaction of fH with both OspE and BBA68 was investigated. The determinants of these proteins involved in binding fH were identified using deletion, random and site-directed mutagenesis. Mutations in widely separated regions of these proteins abolished fH binding indicating that the interaction involves a discontinuous binding site. For OspE, some of the mutants that lost the ability to bind fH had only a single amino acid change. Computer assisted structural analyses revealed that both fH binding proteins contain putative coiled-coil motifs. Site-directed mutagenesis of putative coiled coil (CC) motifs of OspE and BBA68 revealed that these higher order structures are required for fH binding. Antibodies targeting OspE which were generated as part of experimental infection (iAb) with *B. burgdorferi* were also used to analyze the structure of OspE as well as determine regions of OspE which are required for interacting with the iAb. Results utilizing the same OspE recombinant mutants described above reinforced the finding that structure and confirmation are important for OspE/ligand interaction, although iAb and fH do not bind to identical portions of OspE. In summary the data demonstrate that the binding of fH to the *Borrelia* fH binding proteins, OspE and BBA68, involves higher order structural elements (i.e. coiled-coils). These studies advance our understanding of fH binding as a virulence mechanism and facilitate ongoing efforts to utilize fH binding proteins in the development of microbial vaccines.

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Intranasal Vaccination Promotes Sterile Immunity to *Pseudomonas aeruginosa* Pneumonia

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Pseudomonas aeruginosa is a leading cause of hospital-acquired pneumonia, and therefore a serious concern for individuals with compromised immune systems. Although capable of infecting multiple sites, *P. aeruginosa* is most frequently associated with acute infections of the respiratory tract. We have previously shown that a live-attenuated recombinant *Salmonella* oral vaccine to *P. aeruginosa* lipopolysaccharide serogroup O11 O-antigen promotes increased survival and pulmonary bacterial clearance in a mouse pneumonia model. Our present study focused on the efficacy of intranasal (i.n.) delivery of this vaccine to prevent *Pseudomonas* pneumonia. Mice receiving the vaccine elicited high titers of anti-serogroup O11-specific IgG and IgA antibodies in systemic and mucosal immune compartments. Interestingly, IgA, but not IgG, specific for *Pseudomonas* serogroup O11 was detected in the upper respiratory tract (URT). Immunized animals were infected with *P. aeruginosa* serogroup O11 strains 9882-80 or PA103, a cytotoxic derivative. Mice receiving the vaccine were completely protected from challenge doses as high as ten times the 50% lethal dose with either strain. Viable lung counts from immunized mice at 6 and 12 hours post-infection with 9882-80 revealed rapid bacterial clearance from vaccine-immunized animals as compared to the control animals. Passive i.n. transfer of antisera from vaccinated mice, either prior to or 6 hours after infection, was protective to challenge doses ten times the 50% lethal dose for PA103, indicating that immunity is antibody driven and could be given therapeutically. In addition, opsonophagocytic assays with vaccine but not control antisera induced efficient killing to four additional serogroup O11 strains, but not a serogroup O6 strain, suggesting that antibodies induced by the vaccine correlate to a broad level of protection among serogroup O11 isolates. In an attempt to locate the site of bacterial colonization and clearance, PA103 was made luminescent by genomic integration of the *lux* operon for *in vivo* visualization with the IVIS imaging system. Bacteria were observed post-infection in the URT and lungs of control animals at 12 and 24 hours, but were absent in vaccinated mice at both time points. In addition, at 24 hours *Pseudomonas* was not detected in lung homogenates or nasal washes from animals that received the vaccine. Our results indicate that i.n. vaccination with this vaccine is a potent inducer of mucosal and systemic immunity and promotes sterile immunity to *Pseudomonas* respiratory infection by antibody-mediated mechanisms. Furthermore, our results indicate a prominent role for O-antigen-specific IgA antibodies in the URT of this infection model.

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Use of a virulence-associated gene microarray to analyze the changes in *E. histolytica* transcript levels during colonization of the mouse model of amebic colitis.

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The eukaryotic parasite *Entamoeba histolytica* is a major cause of morbidity and mortality worldwide. Disease however occurs in only a minority of infections and requires parasite invasion of the intestinal epithelium. The most common pathology from *E. histolytica* infection is amebic dysentery, but amebic abscesses in liver, lung, and brain also occur. The mechanisms that control the changes from asymptomatic colonization to invasive amebiasis are not well understood but may involve changes in the expression of proteins involved in *E. histolytica* pathogenicity.

We have developed a virulence-associated gene microarray as a tool for investigation of the changes associated with colonization of ameba trophozoites in the mouse model of amebiasis. Among the changes observed was an up regulation of ferredoxin transcripts. Verification of this result by qRT-PCR confirmed that this gene was significantly increased 3-4 fold at 4h post infection in CBA/J mice. This modulation persisted through 24h, 4, 9, and 29 days post inoculation. Also up regulated at 4h by microarray and verified by qRT-PCR was the methionine gamma-lyase transcript. This perhaps indicates that an up-regulation in [Fe–S] cluster biogenesis occurs during infection. Other verified transcripts include those of known virulence associated factors – amebapore A and one of the family encoding the light subunit of the GalNAc inhibitable lectin. Intriguingly a downregulation of the cysteine proteases mRNA's were observed which may reflect the postulated role of these virulence factors in tissue invasion later in disease progression.

The virulence of amebic strains overexpressing these transcripts is being assessed using the mouse model of amebic colitis in both a resistant and susceptible (C57Black/6, and CBA/J) mouse stains.

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Transcription Of The *bhuA* Gene Of *B. abortus* Is Hemin Dependent And Required For Virulence In Experimentally Infected BALB/c Mice

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Brucella abortus is a Gram-negative facultative intracellular pathogen that resides within the phagosomes of host macrophages. The ability to survive and replicate in these macrophages is critical for the establishment of chronic infection. The limited availability of free iron within the macrophage suggests the brucellae must possess mechanisms that will allow them to acquire iron in this iron restrictive environment. Heme and heme containing proteins are relevant iron sources in the macrophages of the reticuloendothelial system that may serve as an iron source to the invading brucellae. To date, no outer membrane iron transport proteins have been characterized for *B. abortus*, however, mutations in the ferrochelatase gene (*hem*H) of *B. abortus* 2308 produce heme auxotroph mutants that can survive with the addition of exogenous hemin, indicating the presence of hemin transport machinery in *B. abortus* 2308. Searches of the *Brucella melitensis* 16M genome reveal the presence of an open reading frame with significant homology to genes encoding the outer membrane hemin receptors of other pathogenic bacteria. The analogous genetic locus was targeted for mutagenesis in *Brucella abortus* 2308 to evaluate the role the corresponding gene product plays in iron acquisition from heme and its contribution to the virulence of B. abortus 2308. Loss of the *bhuA* gene product results in an inability to utilize hemin as an iron source on a chelated medium or in a low iron minimal medium broth. Mutation of the bhuA gene also results in an inability to maintain spleen infection in experimentally infected BALB/c mice. Transcription of the bhuA gene appears to be only marginally iron responsive displaying only slightly increased promoter activity under low iron conditions. However, *bhuA* promoter expression does appear to be hemin dependent displaying no promoter activity in a defined medium without hemin and subsequent promoter activation when hemin is added to the defined medium. Present studies are aimed toward further defining the regulatory link between hemin and *bhuA* promoter activation and to further explore the defect in survival within the BALB/c mouse at the level of the macrophage.

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Molecular and Functional Domain Analysis of *secA* Homolog from Rickettsiae

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Several members of genus *Rickettsia* are responsible for the most severe bacterial diseases of humans. These gram-negative, obligate, intracellular bacteria are transmitted to their mammalian hosts by arthropod vectors such as ticks, fleas and lice
Although the biology of rickettsiae is well known, lack of genetic manipulation system has hampered our ability to characterize the gene function of rickettsiae. Of importance,
the molecular mechanism of protein secretion, an important aspect of bacterial pathogenesis, in rickettsiae remains a major subject of research. Research on various bacterial pathogens has demonstrated that the majority of virulence factors are either secreted into the extracellular environment or attached to the cell surface. In order to elucidate the protein secretion pathways and their involvement in rickettsial pathogenesis, it is important to characterize the genes involved in rickettsial protein secretion. In this study we report the molecular and functional analysis of the putative *secA* gene, an essential component of the Sec-dependent protein secretion pathway, from *Rickettsia rickettsii* and *R. typhi*, the etiologic agents of Rocky Mountain spotted fever and murine typhus, respectively. The sequence analysis of the cloned *secA* from *R. rickettsii* and *R. typhi* show open reading frame of 2721 and 2718 nucleotides, respectively. Alignment of the deduced amino acid sequences reveals the presence of highly conserved amino acid residues and motifs considered to be essential for the ATPase activity of SecA in preprotein translocation. Transcription analysis indicates that *R. rickettsii secA* is expressed monocistronically from the canonical prokaryotic promoter with a transcriptional start point located 32 nucleotides upstream of the *secA* initiation codon. Complementation analysis shows that the full length SecA protein from *R. rickettsii* and *R. typhi* fails to restore growth of *E. coli* strain MM52 *secA51* (ts) at non-permissive temperature (42^{o}C) despite the detection of SecA protein expression by Western blotting. However, the chimeric SecA protein carrying N-terminal 408 amino acids of *R. rickettsii* SecA fused with C-terminal 480 amino acids of *E. coli* SecA restores the growth of *E. coli* strain MM52 *secA51* (ts) at the non-permissive temperature (42^{o}C). These results suggest the amino-terminal ATPase domain is highly conserved, while, the C-terminal domain appears to be species-specific.

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Interleukin-12 is required for the innate host defense against lung colonization by *Pseudomonas aeruginosa*.

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Respiratory infection with *P. aeruginosa* is a prelude to fatal pneumonia in cystic fibrosis (CF). While CF knockout (ko) mice show some similarities to the pathogenesis of the disease in humans, the lung pathology is missing from these mice, indicating that genetic modifiers exist that can increase the inherent resistance level to infection. Using an aerosol infection mouse model, we previously found that the inbred mouse strains can be generally classified into two groups based on lung colonization and mortality: susceptible vs. resistant mice. While DBA/2 mice are susceptible to infection, 129/SV, A/J, BALB/c, C57BL/6, C3H/HeN and FVB mice are not. Furthermore, colonization with *P. aeruginosa* causes a strong induction of interleukin-12 (IL-12) in the resistant but not in DBA/2 mice. IL-12 and tumor necrosis factor-α (TNF-α) co-stimulate the release of interferon-γ (IFN-γ) from natural killer and T lymphocytes, which in turn activates the bactericidal mechanisms in macrophages. While it is clear that IL-12 is of importance to the host defense against intracellular bacteria, little is known about whether IL-12 is needed for lung protection from extracellular pathogens such as *P. aeruginosa*. To dissect the function of this cytokine in lung infection, IL-12p40 ko mice (BALB/c-IL12b^{tm1Jm}) were tested for colonization and mortality in this model. When given the same dose of bacteria as measured in colony forming units immediately after exposure, the IL-12 ko mice, like DBA/2, had a significant increase in viable counts recovered from the lungs at the 6 h time point as compared to the BALB/c or C57BL/6 mice. Within 48 h after infection, 100% mortality was observed in the IL-12 ko and DBA/2 strains, whereas no mortality was seen in the BALB/c or C57BL/6 strains. Intraperitoneal (i.p.) injections of recombinant mouse IL-12p40 homodimer 2 h before and 2 h post infection at a dose of 0.6 μg per mouse cured 67% and 47% of DBA/2, respectively, indicating a protective role for this cytokine. However, no protection was observed in DBA/2 mice with an increased dose of 1.0 μg per mouse. Taken together, IL-12 is required for the innate defense against extracellular bacterial lung infections. IL-12-deficient mice are unable to effectively clear respiratory colonization causing mortality from the *P. aeruginosa* infection. Absence of IL-12 in the resistant mice causes the same phenotypic expression of the susceptibility trait as seen in DBA/2 mice. Treatment of DBA/2 mice with IL-12 during infection with *P. aeruginosa* shows a dose-dependent protection in these susceptible animals. Coupled with two previous studies that show a critical role of TNF-α in the *P. aeruginosa* infection and that IL-12p40 is expressed only in lymphoid cells, our results suggest that IFNγ-dependent activation of alveolar macrophages may be a key portal in the fight against lower respiratory tract colonization by *P. aeruginosa*. A better understanding of the innate lung defense network in mice will lead to the development of novel therapeutics for CF.

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Investigating the Translocation of Pertussis Toxin from the ER to the Cytosol – Evidence for Exploitation of the ERAD Pathway

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Bordetella pertussis produces an exotoxin known as pertussis toxin, an important virulence factor. PT holotoxin comprises one A subunit (S1), bearing the enzymatic activity, associated with a pentameric ring of B subunits. PT is an ADP-ribosyltransferase that modifies several mammalian heterotrimeric G proteins. Several bacterial toxins including cholera toxin and also the plant toxin ricin are believed to undergo retrograde intracellular transport through the Golgi apparatus to the endoplasmic reticulum. The ERAD pathway involves removal of misfolded proteins from the ER and degradation (after return to the cytosol); this is a candidate pathway for exploitation by S1 and other toxins for ER to cytosol transport. In the cytosol ERAD substrate proteins are ubiquitinated at lysine residues, for targeting proteins to the proteasome for degradation. For utilization of this pathway PT must presumably avoid ubiquitination and degradation. We hypothesize that since S1 lacks lysine residues it is credible that ubiquitination may be avoided. We predict that PT toxicity will be reduced by the addition of lysine residues allowing ubiquitination and degradation to occur. Mutant forms of PT were engineered replacing one, two or three arginines with lysine residues, in a variety of locations on S1. Several mutants (with multiple lysine substitutions) have been identified with wildtype *in vitro* enzymatic activity but reduced cellular activity, consistent with our hypothesis. Previous studies have provided evidence that trafficking of PT from the ER as part of the cellular intoxication pathway occurs. CHO cells were stably transfected with constructs containing S1 alone or coupled to a signal peptide that targeted S1 to the ER. Even when directed to the ER, S1 ADP-ribosylated target cytosolic G proteins. However S1 expression levels were significantly greater than cell-associated levels from exogenous PT treatment. To investigate *in vivo* expression and transport of S1 bearing mutations, constructs were engineered with S1 under control of a doxycycline-inducible promoter. S1 expression was controlled in a dose dependant manner in CHO cell transfectants; however, low background expression (uninduced) was observed. This uninduced expression was sufficient to bring about complete ADP-ribosylation, even at levels undetectable by western blot. The addition of the signal peptide had no effect on PT's ability to ADP-ribosylate target G-proteins. From these data we can conclude that PT is able to translocate from the ER to the cytosol, and that very low numbers of S1 molecules are sufficient for full activity. Similar S1 constructs were engineered with single and triple lysine mutations under the inducible promoter in the presence and absence of the signal peptide. These constructs have been transfected into CHO cells and the expression and intracellular activity of these construct is being determined.

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Analysis of the Interaction of the *Borrelia hermsii* Factor H-Binding Protein, FhbA, and Factor H

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In N. America, tick-borne relapsing fever (TBRF) is caused by *Borrelia hermsii*, *B. parkeri*, and *B. turicatae*. We previously demonstrated that some TBRF isolates bind factor H (fH). FH is an important regulator of the alternative complement cascade and serves as a co-factor for the factor I-mediated cleavage of C3b. Cell bound fH was found to be competent to mediate the cleavage of C3b demonstrating the biological relevance of fH binding. We have identified the fH-binding protein of *B. hermsii* YOR isolate and designated it as FhbA. The *fhbA* gene was demonstrated to be single-copy and carried by a 220 kb linear plasmid that is found only in isolates with factor H binding ability. Hybridization analyses indicate that this plasmid may be derived from a smaller plasmid into which foreign DNA carrying the *fhbA* gene was inserted. The nature of the interaction between FhbA and the ligands, fH and infection induced anti-FhbA Ab (infection Ab), was also investigated. Truncations of FhbA were generated and tested for their ability to bind these ligands. Independent truncations from either the N or C terminus abolished fH and infection Ab binding indicating that the binding sites are discontinuous. Random mutagenesis provided additional support for a discontinuous fH-binding site. Alteration of as few as 2 aa residues in different regions of the protein was sufficient to abolish fH binding to FhbA. To identify the domains of fH that bind to FhbA, a series of fH sub-fragments comprised of different short consensus repeats were tested for their ability to bind FhbA. FhbA bound SCRs 1-7 and SCR 16-20. The ability to bind SCRs 1-7, which are buried in the mature fH molecule suggest that FhbA may also bind FHL-1 which is a subfragment of fH generated by alternative splicing of the fH transcript. To further define the structural elements of FhbA that are required for the formation of the discontinuous fH-binding site, comparative sequence analyses and site-directed mutagenesis were performed. The sequence of *fhbA* from the *B. hermsii* MAN isolate was determined and compared to that of the YOR isolate. Both sequence exhibited the potential to form coiled-coils near their C-terminus. To test the role of these coiled-coils in fH binding, site-directed mutagenesis of residues essential for coiled-coil formation was performed. Disruption of the coiled-coils led to the complete elimination of fH binding. The identification and characterization of FhbA and its interaction with fH and iAb provides further insight into the molecular mechanisms of pathogenesis of the relapsing fever spirochetes and enhances our understanding of this widespread virulence mechanism.

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Identification of a Novel Protein (PA0122) from *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an important opportunistic pathogen causing chronic lung infections in cystic fibrosis (CF) and immune compromised patients. *P. aeruginosa* strains that initially infect CF patients usually have phenotypes similar to those in the environment. However, compared to these early isolates, strains from chronic infections have unique phenotypic properties including the over production of the polysaccharide alginate, decreased production of exoproducts, decreased expression of lipopolysaccharide O antigen, and an altered lipid A. It is likely these chronically colonizing strains have alterations in expression of other factors. Here we identify genes that show differential expression; these studies may lead to novel targets for vaccine or drug development, new diagnostic reagents, as well as further our understanding of *P. aeruginosa* pathogenesis.

In this study, *P. aeruginosa* GeneChip Microarray analysis was used to monitor the global gene expression profile in two genetically similar, but phenotypically distinct strains of *P. aeruginosa*: 383 (non-mucoid) and 2192 (mucoid), which were isolated from a single CF patient. Strains were grown in Luria broth at 37°C with shaking to reach mid-log phase (optical density of 0.5 at 600 nm). Based on microarray analysis, several genes were identified that showed differential patterns of expression between these two strains. Among the genes whose expression changed significantly was PA0122. This gene showed a higher expression level (8.02 fold) in 383 compared with 2192. The PA0122 gene is predicted to encode a 136-amino acid "conserved hypothetical protein" with a molecular size of 15 kDa, and p*I* of 4.65. PA0122 protein had limited similarity with known proteins from the databank, but is homologous to the fungal *Aspergillus fumigatus* (Asp)-hemolysin protein and has a motif that matched with the fungal Aegerolysin family of proteins. Protein prediction revealed a 21 amino acids transmembrane region at the N-terminus as well as predicted cleavage site at the C-terminus with short stretch of hydrophobic amino acids. This gene has been cloned from strain 383, and expressed as PA0122-His tagged protein in *Escherichia coli*. This recombinant protein was used for the polyclonal antibody production in mice, and the antibody was used to detect the presence of PA0122 protein in *P. aeruginosa*. Western blotting of 383 revealed PA0122 in the supernatant and as well as in the cell lysate; protein levels were dependent on growth phase and cell density. Using 2-D Western blotting, PA0122 protein has been localized at the similar mass of 16 kDa and p*I* of 4.5, as it was predicted earlier in 383, confirming that PA0122, which had only be recognized by an opening reading frame, encodes a novel protein in *P. aeruginosa.* The presence of PA0122 protein varied among other *P. aeruginosa* isolates including both non-mucoid and mucoid strains. Interestingly, PA103 showed a different protein pattern compared with other strains. Real time-PCR characterization of PA0122 revealed that the gene expression levels correlate with the observed protein expression pattern in 383.

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Pht is where it's at: amino acids signal *Legionella* to differentiate in macrophages

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Whether to make use of local energy sources or to tolerate a range of acidity, osmolarity or temperatures, microbes adjust their physiology via signal transduction pathways. Cellular differentiation is also crucial for pathogens, whose challenge is to colonize one host, then be transmitted to the next. Conspicuous but also technically challenging models of microbial differentiation are *Coxiella burnetii* and *Chlamydia trachomatis,* two obligate intracellular pathogens that shift from parasitic replicative cells to hardy extracellular transmissive forms. To investigate microbial differentiation and its impact on the interplay between intracellular pathogens and their macrophage hosts, we exploit *Legionella pneumophila* as an experimental tool *L. pneumophila* is a gram negative, facultative intracellular pathogen of freshwater amoebae that can also replicate within human alveolar macrophages to cause a severe pneumonia known as Legionnaires' Disease.

As predicted for a microbe that transits between amoebae and water, *L. pneumophila* has a biphasic life cycle. When conditions are favorable for replication, traits that promote transmission are repressed, and the bacteria multiply within phagocyte vacuoles. As nutrients become limiting, the progeny differentiate to the transmissive phase, repressing multiplication while inducing a multitude of traits that equip *L. pneumophila* to escape from its spent host cell, survive as a planktonic cell, and re-establish a replicative niche within a new phagocyte. Mutation of the *phtA* locus of *L. pneumophila* disrupts this phase shift, perturbing differentiation in rich broth and replication in minimal medium and in macrophages. In particular, *phtA* mutants 1) prematurely express certain transmission traits in broth, 2) are transmitted efficiently to macrophages, yet fail to differentiate to the replicative form, 3) resume replication at any point after infection when expression of the wild-type allele is induced or 4) when threonine is provided in dipeptide or amino acid form.

The predicted PhtA protein contains 12 transmembrane domains characteristic of the Major Facilitator Superfamily (MFS) of transporters. *L. pneumophila* strain Lp02 encodes 10 additional homologues of *phtA*, one of which, *phtJ* (*milA*) is also required for intracellular growth. Only the phylogenetically related obligate intracellular pathogen *Coxiella burnetii* contains close homologues to the *pht* family of transporters. We propose a model whereby *Legionella* require the PhtA (*Ph*agosomal *t*ransporter A) protein to acquire the amino acid threonine; by signaling a nutrient rich environment, threonine then triggers differentiation to the replicative form. Extending our analysis to other *pht* family members will provide insight to the mechanisms by which intracellular pathogens sense and respond to their environment.

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Regulation of biofilm development by the *Bordetella* BvgAS signal transduction system

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The *Bordetella* BvgAS locus encodes a two-component system that regulates the expression of nearly all of the known virulence factors synthesized by these gram-negative respiratory pathogens and plays an important role in their survival strategy. In response to incremental changes in the concentrations of environmental signals, BvgAS mediates the control of multiple phenotypic phases and a spectrum of gene expression profiles specific to each phase. Studies highlighting the critical role of this signaling circuitry in the *Bordetella* infectious cycle have focused on planktonically growing bacterial cells. It is becoming increasingly clear that the major mode of bacterial existence in the environment and within the body is a surface-attached state known as biofilms. Biofilms are defined as consortia of sessile microorganisms that are embedded in a matrix. During routine growth of *Bordetella* under agitating conditions, we noticed the formation of a bacterial ring at the air-liquid interface of the culture tubes. We show here that this surface adherence property reflects the ability of these organisms to form biofilms. We further demonstrate that the BvgAS locus regulates biofilm development in the three *Bordetella* species *B. pertussis*, *B. bronchiseptica* and *B. parapertussis*, which differ in their host specificity. By carrying out assays for biofilm formation under both static and dynamic conditions, we show that the Bvg-mediated control in biofilm development is exerted at later time-points, after the initial attachment of bacteria to the different surfaces. Furthermore, we demonstrate the three-dimensional architecture of these biofilms by microscopic analyses. Resistance to antimicrobials is one of the defining biofilm-specific properties that distinguish these surface attached microbial-populations from their planktonic counterparts. We show that these biofilms are highly tolerant to a number of antimicrobials including the ones that are currently recommended for treatment of both veterinary and human infections caused by *Bordetella*. Finally, we propose that the biofilm mode of lifestyle is important in potentially contributing towards persistent infections.

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01

Mutations that Influence Bacteriophage Sensitivity Alter Intracellular Growth and Mouse Virulence of *Listeria monocytogenes*.

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Listeria monocytogenes is a food-borne pathogen that, following ingestion of contaminated food, is able to translocate across the intestinal epithelium to colonize other sites within the body. In order to establish an infection a number of bacterial components and host cell components must interact at the cellular level. In this study we obtained L. monocytogenes Tn917 insertion mutants that were reduced in their ability to form plaques on several cell lines including the human and mouse enterocyte cell lines, CaCo2 and MODE K, respectively. These mutants were identified as being resistant to a derivative of listerial bacteriophage P35. In all cell lines tested, mutant binding efficiency (measured as bacteria bound to monolayers divided by total bacteria added) was similar to the parental. However, the plaquing efficiency (plaques formed/number bacteria bound) and plaque size were dramatically reduced. Intracellular growth rates measured in MODE K cells were similarly drastically reduced. In vivo, competitive index experiments using the parental strain orally inoculated into female A/J mice revealed severe attenuation as measured by decreased mutant colonization in liver and spleen when organs were harvested at 48 h and 96 h post inoculation. Phage binding experiments revealed that the mutant was unable to bind detectable numbers of phage, which is consistent with the hypothesis that the normal phage receptor has been altered due to the insertion mutation. Accordingly, the lesion putatively conferring phage resistance lies in a gene whose predicted product is implicated in proper glycosylation of teichoic acids.

02

Characterization of DhbR, A Transcriptional Activator Of Iron Acquisition Genes In *Brucella abortus*

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Brucellosis is a zoonotic disease caused by the Gram–negative intracellular pathogens comprising the bacterial genus *Brucella*. In the natural ruminant host, brucellosis leads to reproductive failure, manifesting as either abortion, or the birth of severely weakened offspring. In humans, brucellosis results in a long-term, cyclic, flu-like illness known as undulant or Malta fever.

Iron is essential to the survival of *Brucella abortus*, but the mammalian host represents an extremely iron-restricted environment. In response to this restriction, *Brucella* synthesizes two catechol-type siderophores, 2, 3-dihydroxybenzoic acid (DHBA) and the more complex siderophore, brucebactin. Both are produced through the enzymatic activities of the products of the *dhb* operon, and expression of this operon is tightly regulated in response to environmental iron levels. Two consensus Fur boxes are located within the *dhb* promoter, suggesting regulation by Fur, but an isogenic *fur* mutant constructed from *B. abortus* 2308 displays wild-type repression of *dhb* expression in response to iron-replete growth conditions, indicating that an alternate regulator controls expression of the *dhb* operon.

One strategy employed by some bacteria to regulate siderophore biosynthesis is the use of AraC-like transcriptional activators. Examples of these activator proteins are YbtA (yersiniabactin A) of *Yersinia pestis* and AlcR (alcaligin biosynthesis regulator) of *Bordetella bronchiseptica*. In these organisms, the end product siderophore serves as a co-activator in conjunction with the AraC-like protein, and this activation is iron-responsive.

Brucella abortus 2308 possesses a homolog of the *B. bronchiseptica* AlcR. An isogenic *B. abortus alcR* mutant, BEA5, shows decreased catechol siderophore production under iron-deplete conditions, when compared to the parental 2308 strain, suggesting that the product of this gene, termed DhbR (dihydroxybenzoic acid regulator), functions as an activator of siderophore biosynthesis. Additional studies indicate that this regulation occurs at the level of transcription and is the result of direct interactions between DhbR and the *dhb* promoter region.

While DhbR is required to achieve maximal siderophore production, experimental evidence suggests the presence of an additional level of iron-responsive regulation for the *dhb* operon. The nature of the complex interplay between DhbR and other potential iron-responsive transcriptional regulators in controlling expression of the *dhb* operon is currently under investigation.

03

The Role Of Pertussis Toxin In *Bordetella Pertussis* Infection Of Mice.

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Bordetella pertussis is the cause of whooping cough, an acute respiratory infection that can cause severe symptoms and death in young children. An important virulence factor of *B. pertussis* is pertussis toxin (PT), which has been shown to play an early role in colonization of the respiratory tract of mice.
We hypothesized that the reason for this observation is that PT inhibits the early influx of neutrophils to the lungs thereby decreasing or delaying the innate immune response of the host. To test this, we inoculated BALB/c mice with wildtype (WT) and pertussis toxin-deficient (ΔPT) strains, performed bronchioalveolar lavage (BAL) at different time points and determined the number of neutrophils among the cells present in the BAL fluid. Our results showed that, at early time points, mice infected with WT have a significantly lower number of neutrophils in the BAL fluid than mice infected with ΔPT. We speculated that this might be due to an early effect of PT on the secretion of neutrophil-attracting chemokines and cytokines, which we tested by analyzing gene expression in lungs of mice infected with WT and ΔPT using a microarray system. Preliminary data indicate a downregulation by PT of chemokines and cytokines such as IL-6, MIG, LIX, and IFN-γ, all of which are important regulators of early immune responses. This experiment was repeated using BAL macrophages from infected mice instead of whole lungs and our results showed very few differences in chemokine and cytokine production between the two groups of mice, which make it unlikely that macrophages are the main target cells for PT inhibition of neutrophil recruitment. Our hypothesis is that PT inhibits neutrophil influx to the lungs by suppressing the production of neutrophil-attracting cytokines and chemokines by cells other than macrophages that are present in the lungs of mice.

04

Genes for Type IV Pili are Highly Conserved in *Francisella tularensis* Subspecies

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Francisella tularensis is the causative agent of tularemia. Inhalation of as few as 10 organisms can elicit the respiratory form of the disease in humans, and this has let to its classification as a potential agent of biological terrorism. *F. tularensis* is known to be a facultative intracellular pathogen, however, its virulence factors remain largely unknown. A database search of the genomic sequence of the *F. tularensis* Schu4 strain revealed loci with similarity to genes involved in type IV pilus structure and assembly. Type IV pili are associated with pathogenesis in many gram-negative bacteria, and in these organisms variation of the amino acid sequence of the pilin subunits may contribute to evasion of host immune response. Five genes that could potentially encode type IV pilin subunits were identified in *F. tularensis* and designated *pilE1* through *pilE5*. To examine the variability of the pilin subunits among different subspecies of *Francisella*, the DNA and derived protein sequences of the Schu4 genes were compared to sequences from other isolates of *F. tularensis* subsp. *tularensis*, as well as isolates from *F. tularensis* subsp. *holarctica* and the Live Vaccine Strain (LVS), a derivative of *F. tularensis* subsp. *holarctica*. The derived protein sequences were essentially identical between Schu4 and other *F. tularensis* subsp. *tularensis* isolates. Highly similar orthologues of these genes were also present in the other subspecies of *Francisella* though two genes in *F. tularensis* subsp. holarctica and three genes in the LVS did not contain complete open reading frames. Pili-like fibers were observed on the surfaces of both virulent and avirulent strains, and must be further characterized to determine whether they truly represent type IV pili. Constitutive expression of all five pilin encoding genes thus does not appear to be required for piliation, however the conservation of the five pilin subunit genes may indicate importance in the biology of the *Francisella* species.

05

Pseudomonas aeruginosa Outer Membrane Vesicles Export PaAP Aminopeptidase and Activate Human Lung Epithelia

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Pseudomonas aeruginosa is a gram negative, opportunistic pathogen that is a major cause for morbidity and mortality in individuals with compromised lung function such as in patients with cystic fibrosis (CF). One major cause of lung injury results from the acute inflammatory response to the infection. Vesicles, consisting of periplasmic and outer membrane proteins and lipids, are secreted by *P. aeruginosa* as well as many other well-characterized pathogens. We characterized vesicles produced by *P. aeruginosa* and investigated their interactions with human respiratory cells and the consequent immune response. Vesicles from CF strains exhibited the highest association with human lung epithelia. Sequencing of vesicle proteins revealed that the aminopeptidase PaAP (PA2939) was enriched in vesicles from CF strains compared to vesicles from other strains. Furthermore, full-length PaAP was enriched in vesicles compared to periplasmic and outer membrane fractions and PaAP appeared in the supernatant predominantly in a high molecular weight complex (>100 kDa), suggesting it is preferentially exported via vesicles. Vesicle-associated PaAP in vesicles was found to be active and exteriorly located. Since vesicles are likely to come into contact with host cells during an infection, we investigated the cellular response to vesicles from *P. aeruginosa* strains of different origins. The induction of IL-8 by vesicles was significantly higher than that elicited by a 10-fold higher amount of purified LPS from the same strain, suggesting that LPS alone was not responsible for the IL-8 response. These results suggest that *P. aeruginosa* infecting the CF lung produce vesicles enriched in a specific protease that associate with lung cells and can contribute to the inflammatory response.

06

The Role Of AlgR And FimS In The Control Of Twitching Motility In *Pseudomonas Aeruginosa*.

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Pseudomonas aeruginosa is an opportunistic pathogen that can infect immunocompromised individuals and cystic fibrosis patients. This bacterium produces a wide range of virulence factors including the exopolysaccharide alginate and type IV fimbriae. Type IV fimbriae retraction and extension mediate twitching motility, which is a flagella-independent mode of solid surface translocation. Both twitching motility and pilus production are essential for *P. aeruginosa* virulence. The FimS/AlgR sensor-regulator pair regulates the biosynthesis of type IV fimbriae (pili). At present, the mechanism by which AlgR controls twitching motility is unknown. Most response regulators require phosphorylation at an aspartate residue for their activity. AlgR is phosphorylated at Asp54 and mutating this residue results in the loss of twitching motility. Deletions of both *algR* and *fimS* also result in the loss of twitching motility. Western blot analyses on whole cell lysates of *algR* mutants show no defects in pili production. However, transmission electron microscopy and Western blot analyses on surface sheared samples reveal that *algR* mutants lack surface expressed pili. Cellular fractionation studies on the *algR* mutants show that pilin monomers are trapped in the cytoplasmic space and fail to reach the periplasmic space. Thus, while *algR* mutations do not affect pili production, it is clear that AlgR plays an important role in modulating proper pili localization. Data from adherence assays also show that both the *algR* deletion mutant and the *algR* phosphorylation mutant are significantly reduced in their ability to adhere to human bronchial epithelial cells. These findings indicate that *algR* is required for both twitching motility and adherence. Thus, the overall effects of mutating *algR* suggest a decrease in *P. aeruginosa* virulence.

07

Identification of a putative transmembrane kinase with a role in phagocytosis of host cells by *Entamoeba histolytica*

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Entamoeba histolytica is the causative agent of amebiasis, a disease associated with colitis and liver or brain abscesses. This infection is associated with massive tissue damage, which has been shown to involve the induction of apoptotic factors in host cells as well as the ingestion multiple cell types by the ameba. Since phagocytosis has been linked to virulence *in vivo*, the goals of this study were to identify and characterize possible participants in early phagocytosis events. In order to create a proteome of phagocytosis, we incubated ameba with carboxylated magnetic beads in intervals of time. Once beads were ingested, ameba were physically disrupted and intact phagosomes were isolated using a magnet. These phagosomes were delipidated and sequenced by liquid chromatography-mass spectrometry (LC-MS). The results of this analysis proposed the role for one transmembrane kinase (PhAK1) in phagocytosis. This was compelling given that there are around 100 transmembrane kinases present in the *Entamoeba histolytica* genome.

The intracellular portion of PhAK1 (containing the kinase domain) was expressed as a fusion with a 6X HIS tag in *Escherichia coli*. This protein was purified using a nickel agarose bead column and polyclonal serum was raised in NZW rabbits. Unfortunately by western blot this serum has shown to be broadly cross-reactive to between 9 and 11 other transmembrane kinases in *E. histolytica*. However, confocal microscopy using anti-PhAK1-kinase serum has shown that staining only occurred with permeablized cells indicating type 1 integral membrane proteins. This staining appeared as punctate surface staining. Following ingestion of human erythrocytes, the staining surrounded the phagosome. Although we have yet to indict PhAK1 in phagocytosis, these studies are suggestive, and will continue as we build more specific reagents. To date these are the first studies that have proposed a role for transmembrane kinases in the ingestion process of *E. histolytica*.

08

A Two-Component Signal Transduction Response Regulator Binds the Promoter Region of Virulence Genes *ompS* and *htpAB* in *Legionella pneumophila* Philadelphia –1

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Legionella pneumophila (Lpn) is an opportunistic pathogen that causes atypical pneumonia (i.e. Legionnaire's Disease) in susceptible individuals. Lpn is a natural intracellular parasite of aquatic protozoa in which it displays a dimorphic life-cycle, alternating between the replicative form (RF) and the cyst-like mature intracellular form (MIF). It is hypothesized that sodium ion levels in the intracellular milieu of protozoa or alveolar macrophages signals the engulfed MIF to transition into the RF. The Lpn major outer membrane (MOMP) has been implicated in virulence by facilitating attachment to, but not invasion of, host cells. The Lpn heat-shock 60 kDa protein (Hsp60), however, facilitates invasion of HeLa cells and its secretion is dependent on a functional *dot/icm* type IV protein secretion system. Early in intracellular infection of macrophages, MOMP (*ompS*) levels decreased whereas Hsp60 (*htpB*) levels increased suggesting the presence of regulatory factors that respond to environmental signals. Recently, integration host factor (IHF) was found to be upregulated in early stationary phase, and IHF binding sites have been found in the upstream promoter regions of both *ompS* and *htpAB*, of which some of the sites are located in the -35 and -60 regions relative to the transcription start sites. Here we report that functional proteomic analysis revealed a two-component signal transduction system that shared homology to the *E. coli* CpxAR two-component stress response signal transduction system. *In vitro* gel retardation and DNaseI footprint assays with purified His10-Lpn CpxR protein revealed five binding sites in the *ompS* promoter region and two binding sites in the *htpAB* promoter region. The consensus binding site sequence for Lpn CpxR is TGATRRRRRR where R=A or T. Interestingly, the -60 region in the *ompS* promoter region and the -35 region in the *htpAB* promoter region were overlapped by the CpxR binding sites. Taken together, our results suggest that Lpn CpxR response regulator, along with its cognate histidine kinase CpxA, may play a role in the sodium modulatory effect observed on the *ompS* and *htpAB* expression levels.

09

Modulation of EpsE ATP hydrolysis is through interaction with components of the type II secretion system

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The type II protein secretion system Eps in *Vibrio cholerae* facilitates the extracellular secretion of cholera toxin and degradative enzymes through the activity of an envelope spanning apparatus. Characterization of the interactions between proteins present in this apparatus will allow us to better understand the mechanism of type II secretion. We have previously reported that secretion via this multiprotein complex requires the cytoplasmic ATPase EpsE. Although we found purified EpsE to possess low ATPase activity, the activity of EpsE is consistent with ATPases present in other bacterial secretion systems. Previous studies have also indicated interactions between EpsE and the integral inner membrane proteins EpsL and EpsM at the cytoplasmic membrane. EpsF, another integral membrane protein with multiple membrane spanning domains, has also been suggested to participate in the formation of an inner membrane complex with EpsE, EpsL, and EpsM.
In this study we demonstrate that acidic phospholipids and the cytoplasmic domain of EpsL stimulate the ATP hydrolyzing activity of EpsE. In contrast, we find that the cytoplasmic domain of EpsF inhibits the activity of EpsE. Examination of binding interactions using purified EpsE and either EpsL or the cytoplasmic domain of EpsF suggest that these proteins are capable of interacting *in vitro*. We have investigated these interactions further *in vivo* using fluorescent analysis of GFP-tagged Eps proteins and co-immunoprecipitation. Our data suggest a potential regulatory role for both EpsL and EpsF in the coupling of ATP hydrolysis to the type II secretion apparatus.

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Allosteric Stimulatory Effects of BfpC and BfpE on the Zn-ATPase BfpD of the Enteropathogenic *Escherichia coli* Bundle-Forming Pilus Biogenesis Machine

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Although type IV pilus biogenesis, protein secretion, DNA transfer and filamentous phage morphogenesis systems are involved in diverse processes, they are thought to possess similar architectures and mechanisms. These multiprotein complexes include members of the PulE superfamily of putative NTPases that have extensive sequence similarity and probably similar functions as the energizers of macromolecular transport. We have purified the PulE homologue BfpD of the bundle-forming pilus (BFP) biogenesis machine of enteropathogenic *Escherichia coli* and characterized its ATPase activity, providing new insights into its mode of action. Gel filtration and electron microscopy revealed that BfpD forms hexamers in the presence of nucleotide. Hexameric BfpD displayed weak ATPase activity that was highest in the presence of Zn^{2+}. We previously demonstrated that BfpD binds to the cytoplasmic N termini of cytoplasmic membrane proteins BfpC and BfpE. Here, we identified two BfpD-binding sites, $BfpE_{39-76}$ and $BfpE_{77-114}$, in the N terminus of BfpE using a yeast two-hybrid system. Isothermal titration calorimetry and protease sensitivity assays showed that hexameric BfpD-ATPγS binds to $BfpE_{77-114}$, while hexameric BfpD-ADP binds to $BfpE_{39-76}$. Interestingly, the N terminus of BfpC and $BfpE_{77-114}$ together increased the ATPase activity of hexameric BfpD over 1200-fold to a V_{max} of 75.3 μmol P_i min^{-1} mg^{-1}. We conclude that BfpC and BfpE recruit hexameric BfpD to the BFP biogenesis machine and dramatically stimulate its ATPase activity. Furthermore, we propose a model for the mechanism by which BfpD transduces mechanical energy to the BFP biogenesis machine, involving differential nucleotide-dependent binding of hexameric BfpD to $BfpE_{39-76}$ and $BfpE_{77-114}$.

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Alkyl Hydroperoxide Reductase (*ahpC)* Plays a Role in Protecting *Helicobacter pylori* Against Oxidative Stress.

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Helicobacter pylori (*H. pylori*) causes life long infections of the human gastric mucosa. All infected individuals may experience gastritis (inflammation) that can later lead to duodenal and peptic ulcers, MALT lymphoma and gastric cancer. Little is known about the mechanisms employed by *H. pylori* in establishing persistent infections or factors involved in more severe disease. Because inflammation is often associated with the recruitment of phagocytes and release of reactive oxygen species (ROS), oxidative stress proteins such as superoxide dismutase and catalase have been extensively studied. Another protective enzyme, alkyl hydroperoxide reductase (*ahpC*), was determined to be essential for cell viability suggesting that it plays an important role in protection from oxidative stress. The aim of this present study was to determine the role of AhpC in *H. pylori* by modulating AhpC protein levels. An antisense mRNA interference method was constructed to decrease the production of AhpC. A test of the antisense-*ahpC* in an *E. coli* background showed that HP AhpC (plasmid) was decreased over controls. Introduction of an antisense-*ahpC* construct into *H. pylori* (*H. pylori* pDH37::antisense-*ahpC*) also produced a reduction in AhpC levels (SDS-PAGE and immunoblotting). Peroxide challenge studies indicated that the antisense-*ahpC* variant was approximately two-fold more sensitive to H_2O_2 and tert-butyl H_2O_2 than the wild-type in a disk diffusion assay. In summary, AhpC plays a prominent role in protecting *H. pylori* from oxidative stress.

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Intranasal Vaccination Promotes Sterile Immunity to *Pseudomonas aeruginosa* Pneumonia

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Pseudomonas aeruginosa is a leading cause of hospital-acquired pneumonia, and therefore a serious concern for individuals with compromised immune systems. Although capable of infecting multiple sites, *P. aeruginosa* is most frequently associated with acute infections of the respiratory tract. We have previously shown that a live-attenuated recombinant *Salmonella* oral vaccine to *P. aeruginosa* lipopolysaccharide serogroup O11 O-antigen promotes increased survival and pulmonary bacterial clearance in a mouse pneumonia model. Our present study focused on the efficacy of intranasal (i.n.) delivery of this vaccine to prevent *Pseudomonas* pneumonia. Mice receiving the vaccine elicited high titers of anti-serogroup O11-specific IgG and IgA antibodies in systemic and mucosal immune compartments. Interestingly, IgA, but not IgG, specific for *Pseudomonas* serogroup O11 was detected in the upper respiratory tract (URT). Immunized animals were infected with *P. aeruginosa* serogroup O11 strains 9882-80 or PA103, a cytotoxic derivative. Mice receiving the vaccine were completely protected from challenge doses as high as ten times the 50% lethal dose with either strain. Viable lung counts from immunized mice at 6 and 12 hours post-infection with 9882-80 revealed rapid bacterial clearance from vaccine-immunized animals as compared to the control animals. Passive i.n. transfer of antisera from vaccinated mice, either prior to or 6 hours after infection, was protective to challenge doses ten times the 50% lethal dose for PA103, indicating that immunity is antibody driven and could be given therapeutically. In addition, opsonophagocytic assays with vaccine but not control antisera induced efficient killing to four additional serogroup O11 strains, but not a serogroup O6 strain, suggesting that antibodies induced by the vaccine correlate to a broad level of protection among serogroup O11 isolates. In an attempt to locate the site of bacterial colonization and clearance, PA103 was made luminescent by genomic integration of the *lux* operon for *in vivo* visualization with the IVIS imaging system. Bacteria were observed post-infection in the URT and lungs of control animals at 12 and 24 hours, but were absent in vaccinated mice at both time points. In addition, at 24 hours *Pseudomonas* was not detected in lung homogenates or nasal washes from animals that received the vaccine. Our results indicate that i.n. vaccination with this vaccine is a potent inducer of mucosal and systemic immunity and promotes sterile immunity to *Pseudomonas* respiratory infection by antibody-mediated mechanisms. Furthermore, our results indicate a prominent role for O-antigen-specific IgA antibodies in the URT of this infection model.

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Mitogen-Activated Protein Kinases Modulate Cell Cycle Progression in Gastric Epithelial Cells in the Absence or Presence of *Helicobacter pylori* Infection

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Helicobacter pylori infection is associated with alterations of epithelial cell growth and differentiation. It also induces up-regulation of mitogen-activated protein kinases (MAPK) including ERK, p38, and JNK. In other systems, MAPK have been shown to play an important role in mediating cell cycle, proliferation, and differentiation. However, it has not been established whether MAPK modulate gastric epithelial cell cycle in response to *H. pylori* infection. The goal of the current study was to determine if MAPK members differentially regulate cell cycle in gastric epithelial cells in the absence or presence of *H. pylori* infection.

In the absence of *H. pylori* infection, the JNK inhibitor (SP600125) increased ERK and p38 activation in gastric adenocarcinoma cell line, AGS; the p38 inhibitor (SB202190) only increased ERK activation; and ERK inhibitor (PD98059) had no effect on either p38 or JNK activation. Both ERK and JNK inhibitors increased p21 and p27, and had no effect on cyclins A, B. Only JNK inhibitor increased cyclin E and D1 expression. The p38 inhibitor also increased cyclin E, decreased p21 and p27 and had no effect on cyclin A and B and D1 expression. The final results of these changes in the expression of cell cycle machinery are reflected in the modulation of the cell cycle. ERK inhibitor arrested AGS cell cycle at G_1 phase; p38 inhibitor promoted the progression of the cell cycle to the S phase; and JNK inhibitor promoted the progression of the cell cycle to the G_2 phase.

Infection of *H. pylori* (MOI=300:1) resulted in consistent ERK, p38, and JNK activation in AGS cells. *H. pylori* arrested the cell cycle at G_1 phase over 24 hours with an increase in p21, p27, cyclin E and a decrease in cyclins A, B, and D1 expression. In the presence of *H. pylori* infection, JNK inhibitor had little effect on ERK or p38 activation; the p38 inhibitor slightly increased JNK and ERK activation; and ERK inhibitor decreased JNK activation, but not p38 activation. The ERK and p38 inhibitors did not have a consistent or significant effect the *H. pylori* induction of any of the above cell cycle components. At an MOI=150:1, the JNK inhibitor partially restored the levels of cyclin A, B, and D1 expression, and abolished the *H. pylori*-induced cell cycle inhibition, although it had little effect on p21, p27 and cyclin E.

Our data demonstrate that *H. pylori* induced consistent ERK, p38 or JNK activation in gastric epithelial cells. The MAPK members affect one another and coordinately regulate the expression of cell cycle machinery and modulate cell cycle progression. The final outcome of *H. pylori* infection likely depends upon the interaction between bacteria and these MAPK networks.

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A Crp/Fnr-like regulator of Group A *Streptococcus* that significantly influences virulence

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Group A *Streptococcus* (GAS) is characterized by the ability to cause a number of diverse human infections including, pharyngitis, necrotizing fasciitis, toxic shock syndrome, and acute rheumatic fever, yet little is understood about the regulation of streptococcal genes involved in disease processes and survival in the host. Genome scale analysis has revealed a complex regulatory network including 13 two-component regulatory systems and greater than 100 additional putative regulators, the majority of which remain uncharacterized. Here we describe the first regulator of GAS to belong to the Crp/Fnr family of transcriptional regulators. The gene, named *srv* for streptococcal regulator of virulence, encodes a 240 aa protein with 53% amino acid similarity (27% identity) to PrfA, a transcriptional activator of virulence in *Listeria monocytogenes*. Sequencing analysis indicated that Srv is characterized by putative N-terminal β-roll structures which may form an allosteric sensory domain, and a C-terminal helix-turn-helix motif likely involved in the binding of a concensus DNA sequence. Real-time reverse transcriptase-PCR (TaqMan) assays indicated *srv* was transcribed throughout bacterial growth with maximal expression detected in early and late exponential phase. To evaluate the contribution of Srv to GAS virulence, an isogenic mutant strain lacking *srv* and the parental wild-type strain were compared in a mouse model of GAS infection. Mortality was significantly reduced in mice challenged with the mutant strain indicating that Srv is required for full virulence of this pathogen. Complementation in *trans* with *srv* restored the virulence of the mutant strain to wild-type levels. The association of Srv with GAS pathogenesis prompted us to begin to define Srv-regulated genes. A putative Srv-binding site similar to the DNA target sequence recognized by PrfA was identified upstream of 5 GAS genes (*spy1361, spy2007, spy0285, spy0044*, and *spy0714*). TaqMan analysis indicated a decrease in the transcription of each gene in the *srv* mutant strain. Furthermore, recombinant Srv was capable of binding to PCR products containing the binding site upstream of 4/5 genes. As the regulon of Srv my be quite large, a more comprehensive approach is needed to identify Srv-dependent genes. Future experiments will involve the use of SELEX, chromatin immunoprecipitation, and electrophoretic mobility shift assays to identify targets of Srv, while transcriptome and proteome analyses will assess the impact of Srv on GAS gene regulation.

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Characterization Of The Periplasmic Heme Binding Protein, ShuT From *Shigella Dysenteriae*

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Bacterial pathogens require iron as a nutrient for growth and virulence, but iron has poor solubility and is very limited in host cells. Bacteria have evolved several iron acquisition systems to counter these problems, allowing survival in iron-limited environment. General mechanisms for iron acquisition involve siderophore mediated ferric uptake and in bacterial pathogens heme acquisition systems. Typically, gram-negative bacteria utilize a specific outer membrane receptor which belongs to the TonB family of receptors to uptake heme into the periplasm. Once in the periplasm the heme is bound by a periplasmic binding protein, which functions as a soluble receptor for a specific ATP dependent ABC transporter in the inner membrane. There are three distinct families of periplasmic binding protein (PBP). ShuT is most closely related to the BtuB family based on the sequence identities. In this study, we have expressed, purified and characterized the periplasmic heme binding protein, ShuT from *Shigella dysenteriae*. ShuT binds one heme per monomer and the UV/Vis spectrum is typical of a heme bound through a tyrosine residue. Site-directed mutagenesis and spectroscopic analysis have identified Tyr-94 as the ligand to the heme. In addition, we have identified Tyr-228, and Tyr-231 as being important residues in stabilizing the heme within the binding pocket of ShuT. Our present studies will allow us to understand at a molecular level of the mechanism of the heme binding and release.

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Identification of potential proteins involved in *Legionella pneumophila* Hsp60 secretion.

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One of the most abundant proteins synthesized by *Legionella pneumophila*, particularly during growth in a variety of eukaryotic host cells, is a 60 kilodalton member of the GroEL family of molecular chaperons. Previous studies in our lab have established that Hsp60 is mostly located in the periplasm and on the bacterial surface where it promotes invasion of HeLa cells. Analysis of the *L. pneumophila* Hsp60 reveals nothing unique from other members of this highly conserved family of proteins to suggest a mechanism for its secretion. Moreover, the *E. coli* GroEL is also secreted when expressed from a plasmid in *L. pneumophila*, whereas, in *E. coli* both proteins remain in the cytoplasm. A role for known transporters, including types I, II, II, IV, TAT and autotransporters, in secretion of Hsp60 have been systematically eliminated. Nevertheless, we have determined through analysis of mutants of type IV secretion that surface location of Hsp60 is dependent on the Dot/Icm type IV secretion system. However, the mechanisms involved in Hsp60 translocation across the cytoplasmic membrane remain unknown.

The recent annotation of three serogroup 1 *L. pneumophila* genomes provides a good opportunity to identify potential candidates involved in Hsp60 secretion. A large number of putative membrane (or membrane associated) protein-encoding genes have been identified in *L. pneumophila*. Most of theses genes have been ruled out by genomic subtraction with the *Escherichia coli* K12 (Hsp60 non-secreting bacteria) genome and elimination of strain specific genes. The remaining candidate genes have been then reduced to approximately 22 by genomic comparison with *Coxiella burnetii* (Hsp60 secreting bacteria).

The analysis of the *L. pneumophila hsp60* locus indicates that a *dsbD* gene is directly upstream and in the opposite orientation of the *hsp60* encoding operon. This particular organization is very interesting as the Dsb pathway is involved in the folding of periplasmic proteins (by formation and isomerization of disulfide bonds) and *L. pneumophila* Hsp60, as well as the major outer membrane protein OmpS, contains 4 cysteines that could be involved in disulfide bond formation. Genome scanning of *L. pneumophila* has revealed the presence of a unique Dsb pathway composed of a DsbA, a DsbB, two DsbD and one disulfide bond isomerase, CcmG (usually involved in cytochrom C biogenesis). Mutational analysis of this pathway should determine if the Dsb pathway is required for the secretion of Hsp60 and OmpS, two major membrane proteins involved in pathogenesis.

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Characterization of a Host Cytoplasmic Surveillance Pathway Stimulated by Intracellular Bacteria

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A novel pathway has recently been identified that detects the presence of bacteria in the cytosol. Stimulation of this pathway, termed the cytoplasmic surveillance pathway (CSP), leads to activation of interferon regulatory factor 3 (IRF3) and the up-regulation of interferon-β (IFN-β). The CSP does not depend on Toll-like Receptor (TLR) signaling, however, the CSP intersects the MyD88-independent pathway downstream of TLR3 and TLR4. Using the facultatively intracellular pathogen *Listeria monocytogenes* as a model CSP stimulus, we seek to characterize this novel pathway using biochemical and proteomic approaches that involve working backward from the point of intersection with the MyD88-independent pathway. Murine knockouts of the adaptor protein TRIF and siRNA knockdowns of IRF3 kinases TBK1 and IKKε are being used to determine the intersection point of the CSP to the MyD88-independent pathway. Candidate pathway branch point protein interaction partners will be identified using the yeast-two-hybrid system and co-immunoprecipitation / mass spectrometric methods. Interaction candidates will be assessed for their role in the CSP by siRNA knockdown in conjunction with IRF3 activity assays and quantitative real time PCR assessment of IFN-β transcript levels.

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Intracellular cAMP Control Mechanisms Regulate *Pseudomonas aeruginosa* Virulence Gene Expression

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Pseudomonas aeruginosa is an opportunistic human pathogen that can cause life-threatening lung infections in immunocompromised individuals and cystic fibrosis patients. *P. aeruginosa* possesses a wide variety of mechanisms implicated in virulence, including adherence and motility factors (Type IV pili and flagella), toxin expression and delivery systems (Type II and Type III secretion) and quorum sensing. These systems are tightly regulated and expressed in response to specific environmental cues. The transcription factor Vfr (Virulence factor regulator) controls the expression of over 200 genes, including those encoding components of multiple virulence mechanisms. Vfr activity is dependent on the small molecule second messenger adenosine 3', 5'-cyclic monophosphate (cAMP) and is a member of the cAMP receptor protein family. We have previously shown that mutants defective in the production of Vfr or cAMP are attenuated in a mouse model of acute infection, suggesting that regulation of cAMP synthesis may represent an important virulence factor control mechanism. Although the level of intracellular cAMP is elevated under specific conditions (e.g. calcium depletion or high salt), the mechanism by which cAMP levels are controlled in *P. aeruginosa* is poorly understood.

To further characterize the regulation of cAMP synthesis we focused on CyaB function as a possible control point. In *P. aeruginosa* cAMP is synthesized by either of two adenylate cyclase (AC) enzymes CyaA and CyaB. Under *in vitro* growth conditions CyaB is the major source of intracellular cAMP. CyaB has a modular structure consisting of two domains: i) a C-terminal ATP-binding catalytic domain, and ii) an N-terminal membrane-anchoring domain with six membrane-spanning segments termed Membrane-Associated Sensor 2 (MASE2). To study the enzymatic activity of CyaB we purified the predicted catalytic C-terminal domain under native conditions. Preliminary results indicate that this domain has enzymatic activity *in vitro* (~ 6.5 nmol cAMP/mg/min) and that this activity is severely inhibited by the bicarbonate ion. This finding is surprising given that the bicarbonate ion has been shown to directly stimulate cAMP production in related bacterial and mammalian ACs. Over-expression of the soluble C-terminal domain from a broad host range vector resulted in activation of cAMP-dependent genes in both *E. coli* and *P. aeruginosa*, suggesting that this domain is sufficient for catalytic activity. However, deletion of the N-terminal MASE2 domain from chromosomally-encoded *cyaB* resulted in reduced intracellular cAMP, indicating that the membrane-spanning domain plays an important role in enzyme function. We propose that extracellular signals such as those encountered during infection of the human host could be detected *via* the membrane-anchoring domain while intracellular signals such as metabolic status (bicarbonate) may act directly on the catalytic domain.

We also identified a gene encoding a homolog of the *E. coli* cAMP phosphodiesterase (CpdA), which degrades cAMP to AMP. A non-polar deletion of this gene in *P. aeruginosa* strain PAK resulted in altered expression of cAMP-dependent genes, increased basal levels of intracellular cAMP, and reduced growth rate. Overall, our results indicate that cAMP levels are highly regulated in *P. aeruginosa* and can be controlled at both the level of synthesis and degradation.

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Use of a virulence-associated gene microarray to analyze the changes in *E. histolytica* transcript levels during colonization of the mouse model of amebic colitis.

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The eukaryotic parasite *Entamoeba histolytica* is a major cause of morbidity and mortality worldwide. Disease however occurs in only a minority of infections and requires parasite invasion of the intestinal epithelium. The most common pathology from *E. histolytica* infection is amebic dysentery, but amebic abscesses in liver, lung, and brain also occur. The mechanisms that control the changes from asymptomatic colonization to invasive amebiasis are not well understood but may involve changes in the expression of proteins involved in *E. histolytica* pathogenicity. We have developed a virulence-associated gene microarray as a tool for investigation of the changes associated with colonization of ameba trophozoites in the mouse model of amebiasis. Among the changes observed was an up regulation of ferredoxin transcripts. Verification of this result by qRT-PCR confirmed that this gene was significantly increased 3-4 fold at 4h post infection in CBA/J mice. This modulation persisted through 24h, 4, 9, and 29 days post inoculation. Also up regulated at 4h by microarray and verified by qRT-PCR was the methionine gamma-lyase transcript. This perhaps indicates that an up-regulation in [Fe–S] cluster biogenesis occurs during infection. Other verified transcripts include those of known virulence associated factors – amebapore A and one of the family encoding the light subunit of the GalNAc inhibitable lectin. Intriguingly a downregulation of the cysteine proteases mRNA's were observed which may reflect the postulated role of these virulence factors in tissue invasion later in disease progression.

The virulence of amebic strains overexpressing these transcripts is being assessed using the mouse model of amebic colitis in both a resistant and susceptible (C57Black/6, and CBA/J) mouse stains.

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The PhuS Protein of *Pseudomonas Aeruginosa* is a Cytoplasmic Heme-Chaperone Required for Heme Aquisition.

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Iron is essential for the survival and virulence of pathogenic bacteria, but upon entering a host, there is a lack of free iron. Two separate iron acquisition pathways are utilized by bacteria to overcome this problem. These are the siderophore acquisition pathways and the heme uptake systems. In order to utilize heme as an iron source, pathogens have evolved specific transport systems. The transport proteins include an outer membrane receptor, which belongs to the family of Ton-B dependent receptors, which are critical for the uptake of heme into the periplasm. Once in the periplasm, the heme is then bound by a periplasmic binding protein, which mediates heme transfer to the cytoplasm through an ATP-dependent ABC transporter. The cytoplasmic protein in *Yersinia enterocolitica*, HemS, has been shown to be required to prevent heme toxicity. It was originally proposed that the cytoplasmic protein was required for oxidative cleavage of the heme to release the iron for further utilization by the organism. In *Shigella dysenteriae*, the cytoplasmic protein, ShuS, has been shown to bind and sequester heme, but does not exhibit any ability to break down the heme. Since then, heme oxygenases have been found in several pathogenic bacteria including *Neisseriae meningitides, Corynebacterium diphtheriae* and *Pseudomonas aeruginosa.* In the current study we have characterized PhuS from *P. aeruginosa* and its role in heme binding and its involvement in the transfer of heme to heme oxygenase (paHO). In addition we have shown that heme is specifically transferred from PhuS to paHO, and is not transferred to a second HO found within *P. aeruginosa* (BpHO) which has a physiologically distinct role in phytochrome biosynthesis. Therefore, we propose that the PhuS protein is a heme chaperone that functions to sequester and transfer heme to a heme oxygenase (paHO) for further utilization.

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Mutations that Affect Antimicrobial Peptide Resistance Alter Intracellular Growth and Mouse Virulence of *Listeria monocytogenes*

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Listeria monocytogenes is a bacterium that causes severe food-borne illnesses in humans and animals. Once ingested by the mammalian host, the bacteria can translocate across the intestinal epithelium and colonize other sites within the body. Consequences of infection depend, at least in part, on the ability of the bacteria to survive the innate immune defenses of the host. Cationic antimicrobial peptides (CAMPs) are peptides with broad spectrum antimicrobial activity. CAMPs are one means by which plants and animals are protected from the microbes they encounter. An *L. monocytogenes* Tn917 insertion mutant identified as having increased sensitivity to D2A21, a synthetic CAMP, was reduced in its ability to form plaques on several cell lines including the human and mouse enterocyte cell lines CaCo2 and MODE K (respectively). This reduction was not due to a decrease in bacterial binding efficiency (measured as bacteria bound to cell monolayers divided by total bacteria added). Rather, there was a reduction in plaquing efficiency (plaques formed per bacterial cell bound). Also, plaque size was much smaller in the mutant strain. Competitive index experiments performed in orally inoculated female A/J mice revealed that the listerial load in organs harvested at 48 and 96 h post inoculation was significantly reduced from the parental strain. The possible connection between CAMP sensitivity level and virulence was supported in experiments in which two spontaneous D2A21 resistant mutants were isolated and showed higher plaquing efficiencies than the parent and were more virulent in competitive index experiments.

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Analysis of AlgK, A Lipoprotein Required for Alginate Secretion in *Pseudomonas aeruginosa*.

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Chronic pulmonary disease with *Pseudomonas aeruginosa* is a serious contributor to the morbidity and mortality seen in Cystic Fibrosis patients. *P. aeruginosa* isolates from CF patients typically over express an exopolysaccharide termed alginate, which confers upon these bacteria a mucoid phenotype and selective advantages such as resistance to phagocytosis. Alginate is composed of 2 sugar moieties, β-D-mannuronic acid and its C5 epimer, α-L-guluronic acid. Most of the biosynthetic machinery for alginate production is located within the *algD* operon of 12 genes. AlgK is a 52-kDa protein encoded by the fourth gene in the *algD* operon, and it is essential for polymer formation. AlgK mutants secrete degradation products of the newly formed alginate polymers due to the activity of AlgL, an alginate-lyase encoded by the 8th gene in the operon. Thus, AlgK somehow protects new polymers from degradation during transport through the periplasm. AlgK-PhoA fusion proteins are active, indicating that AlgK is localized to the periplasmic space. The N-terminal sequence of AlgK contains a LAAGC motif, which has strong homology to a Type II signal peptide, LXXC, a characteristic of lipoproteins. Studies using E. coli expressing *algK* in trans grown in the presence of ^{14}C palmitic acid showed that AlgK was indeed lipidated. When the conserved Cys residue was mutated to a Ser, resulting in the *algK28* allele, this produced a phenotype in *P. aeruginosa* similar to the AlgK deletion mutant, suggesting that AlgK lipidation is critical for polymer formation. An antibody was raised to purified AlgK, and membrane fractionation studies are being performed to identify the subcellular location of AlgK to either the inner or outer membrane. The finding that periplasmic AlgG (encoded by gene 6 in the operon) is also required to avoid polymer degradation has raised the hypothesis that a multi-protein complex in the periplasm guides alginate secretion. Potential protein-protein interactions involving AlgK and other protein of the alginate secretion machinery are currently being identified by a combination of cross-linking and co-immunoprecipitation studies.

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Overproduction of alginate in a cystic fibrosis isolate of *Pseudomonas aeruginosa* results in reduced virulence in a susceptible mouse model

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Biofilm formation is a leading cause of chronic pneumonia in patients with cystic fibrosis (CF). A capsular material called alginate is important in the formation of biofilms because it provides a structural component for biofilms as seen *in vivo*. While it is clear that overproduction of alginate resulting in a mucoid colony morphology is a clinical hallmark for a poor prognosis in CF, it is unknown about the pathologic role of this overproduced exopolysaccharide during chronic infection. Using an aerosol infection mouse model, we previously identified an inbred mouse strain, DBA/2, which is susceptible to respiratory tract colonization with *P. aeruginosa*. The susceptibility trait is shown with increased bacterial viable counts recovered from the lung of this mouse and mortality. To assess the role of alginate in lung infection, two sets of isogenic strains of *P. aeruginosa* (PAO1 *mucB*$^-$ and PAO1 *algD*$^-$; FRD1 and FRD2) were tested for alginate production, colonization, mortality, cytokine production and histopathology. To knockout the *mucB* gene in PAO1, we created a novel gene inactivation system based on random transposon mutagenesis and homologous recombination. An alginate biochemical assay was performed on all strains to determine the effect of mutations on alginate production. While the *algD*$^-$ and *mucB*$^-$ strains did not show statistically significant difference in colonization and mortality, the cystic fibrosis isolate FRD1 (hyper-mucoid) and FRD2 (nonmucoid due to a suppressor mutation in FRD1) differed significantly in lung colonization and mortality. FRD1 caused an increased bacterial load at 6 h after infection and resulted in only 1 out of 15 DBA/2 mice dead in contrast to 11 out of 16 mice dead for FRD2. This discrepancy may be attributed to the large difference in alginate production between the FRD strains (44 fold) compared with the PAO1 isogenic strains (25 fold). This difference between strains may also rest in the genetic background of FRD and PAO1, as well as the host response to these strains. Interestingly, the reduction of virulence caused by the alginate overproduction in FRD1 can only be seen in the susceptible host. Cytokine expression levels were up-regulated for high alginate strain PAO1 *mucB*$^-$ as well as in low alginate strain FRD2 when compared to their isogenic partner strains. This response supports the notion that overproduction of alginate in FRD1 lowered cytokine expression related to decreased virulence and host inflammatory responses, even with significant lung colonization in DBA/2 mice. FRD originated from a CF patient whereas PAO1 was derived from a burn patient. This explains the response from the FRD1 mucoid strain, such as in a CF environment: reduced virulence is synonymous with overproduction of alginate when colonizing a susceptible host. Taken together, the susceptible mouse model allows a determination of virulence based on alginate production and strain specificity, and can aid in the understanding of chronic colonization of the CF lung. Attenuation in virulence associated with alginate biofilms may benefit the long-term survival of host and pathogen.

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Identification and Analysis of the Avian Pathogenic *Escherichia coli* O78:K80:H9 Virulon

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Poultry diseases caused by avian pathogenic *Escherichia coli* (APEC) cost the American poultry industry over $40,000,000 per year. APEC strains infect chickens, turkeys, ducks, and other avian species via the respiratory tract, contamination of the egg surface, or by systemic infections of the ovary-oviduct. Cellulitis caused by APEC is the leading cause of condemnation of broilers at the time of slaughter. The high stocking density and large numbers of poultry found in the modern broiler industry lends itself to the rapid spread of APEC and as a result, the incidence rate of cellulitis has increased 15-fold over the last several years. While some of the genes required for APEC virulence have been identified, the entire APEC virulon including large virulence plasmids has yet to be identified and the APEC genome has not been sequenced. A complex regulatory network exists in APEC that senses the surrounding environment and activates genes that are required for infection of poultry. The BarA membrane-associated sensor kinase protein and its cognate response regulator, UvrY, are members of an evolutionarily conserved family of two-component signal transduction systems. These systems are composed of a histidine-aspartate kinase protein and a transcriptional regulator protein. In response to these environmental conditions, the BarA-UvrY system controls expression of some virulence factors, changes in metabolism, flagellum biosynthesis, and biofilm formation. Preliminary data in our lab shows that mutations in *barA*, *uvrY*, and *barA-uvrY* affect critical aspects of APEC O78:K80:H9 infection including adherence, colonization, and mortality. Current research is aimed at identifying APEC-specific virulence genes not found in other pathogenic strains of *E. coli* and determining how the BarA-UvrY system regulates their expression *in vivo* during poultry infection. The long-term goal of our research is to develop better intervention strategies for the poultry industry. By targeting APEC factors required for the initial infection process, it may be possible to develop therapies that both prevent APEC O78:K80:H9 disease and prevent infection by other APEC strains.

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NADPH Quinone Reductase FqrB (HP1164) may contribute to Metronidazole Toxicity in *Helicobacter pylori*

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Helicobacter pylori (Hp) lacks the glutathione reductase system found in other bacteria and is dependent on the thioredoxin/ thioredoxin reductase system for providing reducing power for many reactions including activation of alkylhydroperoxide reductase (anti-oxidant defense). Annotation of the two sequenced genomes of Hp revealed two thioredoxin reductase genes, HP0825 (*trxR1*) and HP1164 (*trxR2*) using KE26695 annotation. Here we show that HP1164 TrxR2, which lacks the CXXC catalytic motif required for TrxR activity, exhibits quinone reductase activity. The gene (*fqrB*) was cloned into pET29b and the hexa-his tagged flavoprotein was purified by nickel interaction chromatography following over expression in *E. coli* BL21. Interestingly, IPTG induced expression of *fqrB* in *E. coli*, compared with its un-induced control, increased susceptibility to metronidazole (Mtz) from >250 g/ml to 10 g/ml. The enzyme exhibited a preference for NADPH (over NADH) and reduced a range of substrates including menadione, cytochrome *c*, nitroblue tetrazolium, and weakly reduced DTNB and Mtz. FqrB exhibited a k_{cat} of $139s^{-1}$ and an apparent K_m of 14 M for menadione and 25 M for NADPH. Phylogenetic analysis revealed orthologs of *fqrB* in other species of *Helicobacter* as well as in *Campylobacter jejuni* and *Wolinella succinogenes*, suggesting a divergence from the TrxR family, perhaps necessary for a microaerobic lifestyle. FqrB, like the FrxA nitroreductase of Hp, for which the purified enzyme exhibits little or no Mtz reductase activity, might indirectly reduce Mtz and thereby contribute to the wide range of susceptibilities and resistances to Mtz reported clinically.

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Phenotypic analysis of the alternative sigma factors of *Brucella abortus*

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Brucella abortus is a Gram-negative, facultative intracellular pathogen that causes spontaneous abortions and infertility in domestic animals and a chronic, febrile illness known as undulant fever in humans. The ability of this organism to cause disease is intimately associated with its ability to survive and persist within host professional phagocytes. Once inside the phagosomal compartment of host macrophages, the brucellae are able to resist a variety of harsh conditions, such as exposure to reactive oxygen intermediates, a decrease in pH, and nutrient deprivation. However, little is know about how *B. abortus* is able to survive these conditions and cause disease.
Recent data have suggested that *B. abortus* is able to survive within host macrophages by induction of numerous proteins that are thought to counteract the stresses encountered within the phagosomal compartment. In many organisms, alternative sigma factors of RNA polymerase direct transcription of a distinct subset of genes whose products are involved with survival in various, stressful conditions. The recently sequenced genomes of closely related *B. melitensis* and *B. suis* reveal only six putative sigma factors, of which 4 have unassigned functions. Based on predicted amino acid homologies, we have designated the genes *rpoH1*, *rpoH2*, *rpoE1*, and *rpoE2*. Phenotypic analyses of isogenic mutants in these genes are being employed to determine the role of these gene products in the heat shock and periplasmic stress responses. The ability of these mutants to establish a chronic infection within experimentally infected animals will also be determined. This will allow us to determine the importance of these putative alternative sigma factors in virulence *of B. abortus*.

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Identification and Characterization of the Two AP Endonuclease Homologs of *Brucella abortus* in Resistance to Oxidative Stress.

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Brucella abortus is a facultative intracellular pathogen that is the etiological agent of brucellosis, a disease that is characterized by abortion and infertility in ruminant animals and undulant fever in humans. In the natural hosts and in humans, it is believed that the brucellae establish a chronic infection by surviving within host macrophages for an extended period of time. Upon phagocytosis, the brucellae are able to reside within the phagosome and survive exposure to low pH, nutrient deprivation, and exposure to reactive oxygen intermediates (ROIs), including superoxide radicals, hydrogen peroxide, and hydroxyl radicals. However, to date, little is known about how the brucellae are able to survive the oxidative environment found within the host macrophage.

In bacteria, there are multiple DNA repair pathways to counteract DNA damage incurred by ROIs generated either by endogenous metabolism or by host phagocytes. One such pathway, termed base excision repair, centers around the action of AP endonuclease, which is necessary for the removal and eventual replacement of oxidatively damaged nucleotides within the chromosome. In *Escherichia coli*, the gene that encodes for the predominant AP endonuclease is *xthA*. An *E. coli xthA* mutant exhibits hypersensitivity to hydrogen peroxide suggesting that XthA is necessary for the repair of oxidatively damaged DNA. Furthermore, in the intracellular pathogen, *Salmonella typhimurium*, studies have shown that an AP endonuclease-deficient strain is attenuated in the mouse model, demonstrating the importance of base excision repair *in vivo*.

There are two *xthA* homologs found with the sequenced genomes of closely-related *B. melitensis* and *B. suis*, which have been designated *xthA1* and *xthA2* The *B. abortus xthA2* mutant was created and has shown sensitivities to exposure to hydrogen peroxide, peroxynitrite and DNA damaging agents, such as methyl methanesulfonate, that are consistent with the role of XthA in base excision repair. However this mutant is not attenuated within experimentally infected mice or within infected cultured peritoneal murine macrophages. Since the *xthA2* gene product appears to play a role in resistance to oxidative stress *in vitro* but not *in vivo*, this raises the question of whether the *xthA1* gene product has overlapping function with regard to XthA2 and repair of oxidatively damaged DNA. Ongoing research is focused on answering what role the *xthA1* gene product plays in resistance to oxidative stress.

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Analysis of the Interaction of the *Borrelia hermsii* Factor H-Binding Protein, FhbA, and Factor H

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In N. America, tick-borne relapsing fever (TBRF) is caused by *Borrelia hermsii*, *B. parkeri*, and *B. turicatae*. We previously demonstrated that some TBRF isolates bind factor H (fH). FH is an important regulator of the alternative complement cascade and serves as a co-factor for the factor I-mediated cleavage of C3b. Cell bound fH was found to be competent to mediate the cleavage of C3b demonstrating the biological relevance of fH binding. We have identified the fH-binding protein of *B. hermsii* YOR isolate and designated it as FhbA. The *fhbA* gene was demonstrated to be single-copy and carried by a 220 kb linear plasmid that is found only in isolates with factor H binding ability. Hybridization analyses indicate that this plasmid may be derived from a smaller plasmid into which foreign DNA carrying the *fhbA* gene was inserted. The nature of the interaction between FhbA and the ligands, fH and infection induced anti-FhbA Ab (infection Ab), was also investigated. Truncations of FhbA were generated and tested for their ability to bind these ligands. Independent truncations from either the N or C terminus abolished fH and infection Ab binding indicating that the binding sites are discontinuous. Random mutagenesis provided additional support for a discontinuous fH-binding site. Alteration of as few as 2 aa residues in different regions of the protein was sufficient to abolish fH binding to FhbA. To identify the domains of fH that bind to FhbA, a series of fH sub-fragments comprised of different short consensus repeats were tested for their ability to bind FhbA. FhbA bound SCRs 1-7 and SCR 16-20. The ability to bind SCRs 1-7, which are buried in the mature fH molecule suggest that FhbA may also bind FHL-1 which is a subfragment of fH generated by alternative splicing of the fH transcript. To further define the structural elements of FhbA that are required for the formation of the discontinuous fH-binding site, comparative sequence analyses and site-directed mutagenesis were performed. The sequence of *fhbA* from the *B. hermsii* MAN isolate was determined and compared to that of the YOR isolate. Both sequence exhibited the potential to form coiled-coils near their C-terminus. To test the role of these coiled-coils in fH binding, site-directed mutagenesis of residues essential for coiled-coil formation was performed. Disruption of the coiled-coils led to the complete elimination of fH binding. The identification and characterization of FhbA and its interaction with fH and iAb provides further insight into the molecular mechanisms of pathogenesis of the relapsing fever spirochetes and enhances our understanding of this widespread virulence mechanism.

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Genetic and Environmental Control of Outer Membrane Vesicle Production in *Escherichia coli*

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All Gram-negative bacteria studied to date produce outer membrane vesicles. We propose that the quantity of outer membrane vesicles produced may be regulated in response to changing environmental conditions. Conditions that induce vesicle regulation, and genes that alter the quantity of vesicles produced have yet to be described. To better understand the mechanism and function of vesicle formation, we generated vesiculation mutants using transposon-mediated mutagenesis of *Escherichia coli*. Genes involved in vesiculation identified in this screen include cell wall biosynthesis genes and genes that mediate the envelope stress response. Vesiculation phenotypes were not predictive of membrane defects in mutant strains. Both peptidoglycan biosynthesis and the envelope stress response are regulated during growth phase. To determine if vesicle production also changes with growth phase, samples were taken at several stages from a growing culture and vesicles quantified. The relative rate of vesicle production is highest in early log phase and drops in stationary phase. In addition to alterations in environmental conditions due to growth phase, the cell envelope must also adapt to changes in temperature. To maintain membrane fluidity during cold shock, *E. coli* incorporates unsaturated lipids into the outer membrane. To determine if vesicles contribute to membrane turnover during cold shock, vesicle production was measured from cultures at 12°C and 30°C. Cultures shifted to 12°C exhibited higher vesicle production per CFU from 1 to 4 hours after shift as compared to cultures left at 30°C, but these differences were primarily due to differences in growth rate at the temperatures tested. During heat stress, *E. coli* secretes protein folding and degradation factors to the periplasm. To determine if vesicles contribute to cell envelope turnover during heat shock, vesicle production was measured from cultures at 37°C, 41°C, and 43°C. Cultures grown under heat stress conditions produced more vesicles than control cultures grown at 37°C. Similar to cold temperatures, the effect of heat on vesicle production was dependent on the growth phase of the cultures, and log phase cultures did not increase vesicle production in response to heat. Of the 28 vesiculation mutants identified in our screen, 27 strains exhibited temperature sensitivity at 44°C. These results suggest that alterations in the quantity of vesicles produced may be important to bacterial survival during a variety of environmental challenges.

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The transcriptional activator PqsR and the *Pseudomonas* Quinolone Signal are required for *pqsA* transcription in *Pseudomonas aeruginosa*.

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Pseudomonas aeruginosa is a Gram-negative, opportunistic human pathogen that affects immunocompromised individuals. This bacterium is found in severe nosocomial infections and chronic infections in cystic fibrosis patients. *P. aeruginosa* cell-to-cell signaling controls many virulence factors in a cell density dependent manner, known as quorum sensing. There are two interconnected quorum sensing systems in *P. aeruginosa*, known as the *las* and *rhl* systems. Each system contains a transcriptional activator protein (R protein), encoded by *lasR* and *rhlR* respectively, and their corresponding acyl-homoserine lactone autoinducer; N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) and N-(butanoyl)-L-homoserine lactone (C_4-HSL), encoded by *lasI* and *rhlI* respectively. A third intercellular signal, 2-heptyl-3-hydroxy-4-quinolone, [referred to as the *Pseudomonas* Quinolone Signal (PQS)], is also part of the quorum sensing of *P. aeruginosa*. The PQS biosynthetic gene cluster is made up of *pqsABCDE*, *phnAB* and the transcriptional regulator *pqsR*. Our work has focused on the activation of *pqsA* and the involvement of PqsR. Using beta-galactosidase assays with a plasmid containing *pqsA-lacZ*, we confirmed existing data that show PqsR is required for *pqsA* transcription. We also showed that PQS is required in the presence of PqsR for this activation, and in the presence of a *pqsR* mutant, PQS is unable to activate *pqsA*. Further transcriptional fusion studies assays confirmed existing data that the *las* system is required for *pqsA* transcription and the *rhl* system represses *pqsA*. We also noticed that the addition of PQS to *las* mutants increased the activation of *pqsA*. Construction of a plasmid that over-expresses PqsR in the presence of *pqsA-lacZ* showed more interesting results. We observed the activation of *pqsA* only when PqsR is present, without the presence of PQS, which showed that PqsR can have at least some partial activation alone. This plasmid construct was able to restore *pqsA* activation in a *pqsR* mutant and also allowed *pqsA* transcription in a *lasR* mutant. However, it appears that over-expression of PqsR can cause repression of *pqsA* transcription and so PqsR may have a dual role of repressor and activator.

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A role for flagellin in the pathogenesis of *Bordetella bronchiseptica*

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Species from the genus *Bordetella*, namely *B. pertussis, B. parapertussis* and *B. bronchiseptica*, colonize the respiratory tract of mammals. While *B. pertussis* has adapted exclusively to the human host, *B. parapertussis* can infect either humans or sheep. By contrast with the more host-restricted *Bordetella* spp., *B. bronchiseptica* causes infections in a wide variety of hosts, both human and animal. A two-component signal transduction system, BvgAS, controls a phenotypic transition involving changes in the expression of virulence factors critical in the pathogenesis of *Bordetellae.* Because the function of BvgAS is highly conserved within and across the *Bordetella* genus this phenotypic modulation may constitute for these microorganisms an important adaptive response to either the host or environmental conditions. Interestingly, the Bvg- phase of *B. bronchiseptica* is characterized specifically by the expression of flagellin and motility and the repression of adhesins and toxins necessary for the colonization of the respiratory tract. Our work and that of others has shown that flagellin from *Pseudomonas* and *Salmonella* spp. is an important pro-inflammatory mediator for host epithelial cells. A wide host range and the phenotypic modulation by the BvgAS system makes *B. bronchiseptica* an excellent model to elucidate the role of virulence factors in *Bordetellae* pathogenesis. Thus, we have studied the role of *B. bronchiseptica* flagellin in the development of pro-inflammatory and host defense responses in the airways upon infection. We have found that *B. bronchiseptica* flagellin is a virulence factor critically responsible for the up-regulation of cytokine, chemokine, and host defense gene expression in human lung epithelial cells. In addition, *B. bronchiseptica* flagellin was able to induce NFκB activation *in vitro* and showed potency similar to flagellin isolated from other bacterial species. However, the development of host defense responses in non human lung epithelial cells, as well as in an *in vivo* model of lung infection in mice, was not critically dependent on the expression of flagellin by *B. bronchiseptica*. Our results show that *B. bronchiseptica* flagellin is a potent pro-inflammatory virulence factor for human airway epithelial cells, and suggest the existence of a specific host species tropism in the responses to *Bordetella* flagellin.

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Analysis of Bacterial Synergy in Polymicrobial Infections using a Type II Diabetic Mouse Model.

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Human diabetics frequently suffer delayed wound healing, increased susceptibility to localized and systemic infections, and limb amputations as a consequence of the disease. These infections are often polymicrobial in nature, involving mixtures of aerobic, facultative, and anaerobic bacteria. These bacteria frequently show synergy when inoculated together in animal models that survey abscess formation. Synergy is defined as a statistically significant increase in the number of bacteria in an infection with a second bacterium, as compared to that bacterium alone. The synergy has been hypothesized to depend on factors such as the ability to lower the oxygen concentration of host tissues, synthesis of a capsule, and production of cytotoxins. The purpose of this study is to determine if *Escherichia coli*, *Bacteroides fragilis*, and *Clostridium perfringens* contribute to synergy in polymicrobial infections using type II diabetic mice as an in vivo model. The strain of mice we used for these experiments is BKS.Cg-*m* +/+ *Lepr*^{db}. Two populations of mice were compared: young (5-6 weeks old) prediabetic mice and aged (23-24 weeks old) mice that exhibit hyperglycemia by 4-6 weeks of age. Mice of strain C57BLK/J of the same age were used as non-diabetic controls. Our dual hypothesis is that each bacterial species will be synergistic in the polymicrobial infections and that diabetes and not age is a factor in susceptibility to polymicrobial infections. The mice were injected subcutaneously in the inner thigh with single and mixed cultures containing 1 x 10^6 each of *E. coli*, *B. fragilis*, and *C. perfringens* in all possible combinations. Progression of the infection was monitored by examining bacteriological and immunological indicators at 1, 8, and 22 days post-infection. Abscess formation was monitored and the area of abscess formation was examined for pathological changes during the course of the infection. Synergy was observed between *E. coli* and *B. fragilis* in both the young group and aged group of mice. The aged mice carried a higher bacterial burden than the young mice. No difference in bacterial counts was seen in the control strain of mice, giving support to our hypothesis that age is not a factor in the susceptibility to polymicrobial infections. Work is underway to develop chromosomal mutants of two key virulence factors and the three terminal oxidases of the *E. coli* strain to determine if they play a role in synergy.

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Role of Outer Membrane Vesiculation in the Response to Envelope Stress in *Escherichia coli*

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Outer membrane vesicles are generated by all Gram-negative bacteria studied to date. These vesicles are involved in a variety of bacterial processes including growth, communication, and virulence factor transmission. Despite their ubiquitous nature, little is known about the process by which vesicles are formed. To study the regulatory mechanisms involved in vesicle production and further elucidate the physiological functions of vesicle release, we conducted a Tn5 transposon mutagenesis screen of a laboratory DH5α *E. coli* strain to identify mutants that either over- or under-produced vesicles. Characterization of identified vesiculation mutants has shown no distinct correlation among detergent sensitivity, periplasmic and cytosolic leakiness, viability, and vesiculation of the mutant strains, demonstrating that vesicle production is not a function of membrane integrity. A subset of the mutants identified have disruptions in components of the σ^E stress response pathway, which is responsible for maintaining the envelope under conditions affecting outer membrane protein stability. We have found that disruptions of this pathway causing either constitutive activation or impaired function result in increased vesicle release. Further linking vesiculation and envelope stress conditions, the overvesiculation phenotype of a DegP null mutant is stress-dependent, and increased vesiculation in wild-type cells can be induced by direct activation of the σ^E pathway.

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Demonstration of the involvement of putative coiled-coil domains of the *Borrelia* Factor H binding proteins in interacting with human Factor H

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Lyme disease (LD) is the most common tick-borne zoonosis in the United States with approximately 24,000 cases reported annually to the CDC. LD is caused by the spirochete *Borrelia burgdorferi*, which utilizes multiple mechanisms for immune evasion and is capable of establishing a chronic infection in the absence of treatment. It has recently been demonstrated that these bacteria express two classes of proteins which can bind human factor H (fH); these include the OspE paralogs and BBA68. fH is the second most highly abundant serum protein and is an important regulator of the alternative complement cascade. Several human pathogens have been shown to bind fH to their surface, a process that facilitates immune evasion or cell to cell interaction. The molecular basis of the interaction of fH with both OspE and BBA68 was investigated. The determinants of these proteins involved in binding fH were identified using deletion, random and site-directed mutagenesis. Mutations in widely separated regions of these proteins abolished fH binding indicating that the interaction involves a discontinuous binding site. For OspE, some of the mutants that lost the ability to bind fH had only a single amino acid change. Computer assisted structural analyses revealed that both fH binding proteins contain putative coiled-coil motifs. Site-directed mutagenesis of putative coiled coil (CC) motifs of OspE and BBA68 revealed that these higher order structures are required for fH binding. Antibodies targeting OspE which were generated as part of experimental infection (iAb) with *B. burgdorferi* were also used to analyze the structure of OspE as well as determine regions of OspE which are required for interacting with the iAb. Results utilizing the same OspE recombinant mutants described above reinforced the finding that structure and confirmation are important for OspE/ligand interaction, although iAb and fH do not bind to identical portions of OspE. In summary the data demonstrate that the binding of fH to the *Borrelia* fH binding proteins, OspE and BBA68, involves higher order structural elements (i.e. coiled-coils). These studies advance our understanding of fH binding as a virulence mechanism and facilitate ongoing efforts to utilize fH binding proteins in the development of microbial vaccines.

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Identification of Ca^{2+}-dependent URE3-BP associated proteins in *Entamoeba histolytica*

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Entamoeba histolytica is the second leading cause of parasitic morbidity worldwide. Transcriptional regulation of the parasite's virulence factors is an important determinant of the organism's virulence and adaptation processes. URE3-BP is an EF-hand motif-containing transcription factor that specifically binds to URE3, a regulatory motif present in the promoter regions of the genes encoding two important virulence factors: the lectin heavy subunit (*hgl5*) and ferredoxin (*fdx*). Ca^{2+} has been demonstrated to regulate URE3-BP DNA-binding activity *in vivo* and *in vitro*. To determine whether this effect was due to specific binding of Ca^{2+} to URE3-BP, ^{45}Ca-overlay assays of immunoprecipitated URE3-BP were performed. URE3-BP was demonstrated to directly bind Ca^{2+}. Previous studies using immunoflourescence confocal microscopy to determine the cellular location(s) of URE3-BP determined that the protein was present in the nucleus and cytoplasm and was associated with the plasma membranes of trophozoites. To determine whether URE3-BP differentially associates with other molecules in a Ca^{2+}-dependent manner, immunoprecipitations were performed to "pull-down" URE3-BP associated proteins, and such molecules were further investigated *via* far-western blot analysis in calcium-enriched and -depleted conditions. A protein band with an apparent molecular weight of 22 kDa was identified that only associated with URE3-BP in calcium-enriched conditions. Mass spectrometry to determine the identity of the protein band identified various candidate proteins, however, the most abundant were a protein containing a C2 domain (32.m00217, TIGR *E. histolytica* Genome Project) and a protein homologous to ARPC4 (195.m00084), a member of the Arp2/3 complex. Studies are currently being performed to confirm the identity of the associated molecule(s) to further understand the mechanisms involving calcium regulation of URE3-BP activity.

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Structure/Function Analysis of Alg8, A Putative Glycosyltransferase For Alginate Biosynthesis in *Pseudomonas aeruginosa*.

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Chronic pulmonary infection by *Pseudomonas aeruginosa* is a serious complication for Cystic Fibrosis patients. Alginate, a capsule-like expoloysaccharide, is an important virulence factor of CF clinical isolates. Alginate is composed of D-mannuronate and its C5 epimer, L-guluronate, with beta, 1-4 linkages. The genes for alginate are located in an 18 kb operon that is involved in the biosynthesis, polymerization and secretion of alginate. Alg8, encoded by the second gene in the alginate biosynthetic operon, shows structural similarity to beta-glycosyltransferases and is the putative enzyme involved in the polymerization of D-mannuronate from GDP-mannose. Beta-glycosyltransferases are often inner membrane proteins with a large, cytoplasmic hydrophilic loop that contains two enzymatic domains (A and B). To determine if Alg8 fits this pattern, a TMpred and hydrophobicity plot analysis was performed, which predicted that Alg8 has four trans-membrane domains plus a signal peptide for secretion into the inner membrane. To determine the cellular locations of Alg8's 4 hydrophilic loop regions, translational gene fusions were constructed to a promoterless *phoA* cassette. PhoA fusions at amino acids 37 and 420 in loops 1 and 3, respectively, were positive for alkaline phosphatase activity indicating a periplasmic location. PhoA fusion made to full length Alg8 was also positive for alkaline phosphatase activity. In contrast, PhoA fusions at amino acid 248 and 477 in loops 2 and 4, respectively, were negative for alkaline phosphatase activity and verified by immunoblot analysis. These results suggest that Alg8 is a cytoplasmic membrane protein that spans the membrane four times, and approximately 60% of the protein consists of a large cytoplasmic loop containing the enzymatic domains A and B. In addition, a mutant analysis of Alg8, in which the C-terminus from periplasmic loop 3 was deleted, suggests an important role for this domain in alginate biosynthesis even though the enzymatic domain is intact.

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The BvgAS signal transduction system regulates biofilm development in *Bordetella*

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The majority of *Bordetella* virulence determinants are regulated by the BvgAS signal transduction system. BvgAS mediates the control of multiple phenotypic phases and a spectrum of gene expression profiles specific to each phase, in response to incremental changes in the concentrations of environmental signals. Studies highlighting the critical role of this signaling circuitry in the *Bordetella* infectious cycle have focused on planktonically growing bacterial cells. It is becoming increasingly clear that the major mode of bacterial existence in the environment and within the body is a surface-attached state known as biofilms. Biofilms are defined as a consortium of sessile microorganisms that are embedded in a matrix. During routine growth of *Bordetella* under agitating conditions, we noticed the formation of a bacterial ring around the surface of the culture tubes. We show here that this surface adherence property reflects the ability of these organisms to form biofilms. Our data demonstrate that the BvgAS locus regulates biofilm development in *Bordetella*. The results reported in this study suggest that the Bvg-mediated control in biofilm development is exerted at later time-points after the initial attachment of bacteria to the different surfaces. Additionally, we show that these biofilms are highly tolerant to a number of antimicrobials including the ones that are currently recommended for treatment of both veterinary and human infections caused by *Bordetella*.

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The processed form of LRP1B binds Exotoxin A from Pseudomonas and serves as a receptor for this toxin

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Exotoxin A (PE) from *Pseudomonas aeruginosa* uses LRP1, a multi-ligand receptor, as its cell surface receptor to gain access to cell interior of mammalian cells. The interaction between toxin and this receptor is mediated via structural domain I of the toxin and domain IV of LRP1. Recently, a related receptor, LRP1B, sharing 59% sequence homology with LRP1, was reported to bind some but not all of the same ligands as LRP1. It was therefore an open question as to whether LRP1B could serve as a cell surface receptor for PE. $LRP1^{-neg}$-toxin resistant cells were permanently transfected with a minireceptor form of LRP1B and then challenged with various concentrations of PE. The presence of LRP1B increased toxin sensitivity by at least 100-fold, confirming the functionality of LRP1B as a toxin receptor. In addition, ligand blots of crude membranes from transfected cells indicated that PE interacted preferentially with a 98kDa processed form of the minireceptor, while an antibody to an N-terminal HA tag reacted with both the full length (148kDa) and processed (98kDa) forms of LRP1B. Controls, employing a vector-only transfected cell line or a binding defective mutant of PE confirmed the specificity of these interactions.

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Transcription Of The *bhuA* Gene Of *B. abortus* Is Hemin Dependent And Required For Virulence In Experimentally Infected BALB/c Mice

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Brucella abortus is a Gram-negative facultative intracellular pathogen that resides within the phagosomes of host macrophages. The ability to survive and replicate in these macrophages is critical for the establishment of chronic infection. The limited availability of free iron within the macrophage suggests the brucellae must possess mechanisms that will allow them to acquire iron in this iron restrictive environment. Heme and heme containing proteins are relevant iron sources in the macrophages of the reticuloendothelial system that may serve as an iron source to the invading brucellae. To date, no outer membrane iron transport proteins have been characterized for *B. abortus*, however, mutations in the ferrochelatase gene (*hem*H) of *B. abortus* 2308 produce heme auxotroph mutants that can survive with the addition of exogenous hemin, indicating the presence of hemin transport machinery in *B. abortus* 2308. Searches of the *Brucella melitensis* 16M genome reveal the presence of an open reading frame with significant homology to genes encoding the outer membrane hemin receptors of other pathogenic bacteria. The analogous genetic locus was targeted for mutagenesis in *Brucella abortus* 2308 to evaluate the role the corresponding gene product plays in iron acquisition from heme and its contribution to the virulence of B. abortus 2308. Loss of the *bhuA* gene product results in an inability to utilize hemin as an iron source on a chelated medium or in a low iron minimal medium broth. Mutation of the bhuA gene also results in an inability to maintain spleen infection in experimentally infected BALB/c mice. Transcription of the bhuA gene appears to be only marginally iron responsive displaying only slightly increased promoter activity under low iron conditions. However, *bhuA* promoter expression does appear to be hemin dependent displaying no promoter activity in a defined medium without hemin and subsequent promoter activation when hemin is added to the defined medium. Present studies are aimed toward further defining the regulatory link between hemin and *bhuA* promoter activation and to further explore the defect in survival within the BALB/c mouse at the level of the macrophage.

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Evidence of Pertussis Toxin Transport to the Golgi Apparatus

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Pertussis toxin (PT) is an important virulence factor of *Bordetella pertussis*, the causative agent of whooping cough. The A subunit of PT, known as S1, ADP-ribosylates heterotrimeric G proteins on the cytosolic side of the host cell plasma membrane, leading to the disruption of cell signaling. The B oligomer of PT, consisting of S2, S3, two copies of S4, and S5, binds glycoconjugate receptors on mammalian cells. The current hypothesis is that the route by which PT reaches its target proteins includes a retrograde transport pathway, i.e. via an endosome to the Golgi apparatus and then to the endoplasmic reticulum (ER), before translocation of S1 to the cytosol. In order to test this hypothesis, amino acid residues that are targets for modification in specific cellular organelles were added to subunits of PT. Because tyrosine sulfation occurs in the Golgi, a peptide sequence that can serve as a target for such sulfation was added to either the S1 or S2 subunit. Similarly, a sequence at which N-glycosylation (an ER-specific activity) can occur was also added. Through incubation of mammalian cells with radiolabeled sulfate, followed by incubation with peptide-tagged PT constructs, cell lysis, immunoprecipitation, and analysis by SDS-PAGE and fluorography, evidence for PT transport via the retrograde pathway can be obtained. Tyrosine sulfation of the peptide-tagged S1 and S2 constructs was observed, providing evidence that A and B subunits of PT travel through the Golgi apparatus. Higher molecular-weight forms of these proteins, which would suggest that N-glycosylation occurred in the ER, have not yet been observed.

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Molecular and Functional Domain Analysis of *secA* Homolog from Rickettsiae

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Several members of genus *Rickettsia* are responsible for the most severe bacterial diseases of humans. These gram-negative, obligate, intracellular bacteria are transmitted to their mammalian hosts by arthropod vectors such as ticks, fleas and lice

Although the biology of rickettsiae is well known, lack of genetic manipulation system has hampered our ability to characterize the gene function of rickettsiae. Of importance,

the molecular mechanism of protein secretion, an important aspect of bacterial pathogenesis, in rickettsiae remains a major subject of research. Research on various bacterial pathogens has demonstrated that the majority of virulence factors are either secreted into the extracellular environment or attached to the cell surface. In order to elucidate the protein secretion pathways and their involvement in rickettsial pathogenesis, it is important to characterize the genes involved in rickettsial protein secretion. In this study we report the molecular and functional analysis of the putative *secA* gene, an essential component of the Sec-dependent protein secretion pathway, from *Rickettsia rickettsii* and *R. typhi*, the etiologic agents of Rocky Mountain spotted fever and murine typhus, respectively. The sequence analysis of the cloned *secA* from *R. rickettsii* and *R. typhi* show open reading frame of 2721 and 2718 nucleotides, respectively. Alignment of the deduced amino acid sequences reveals the presence of highly conserved amino acid residues and motifs considered to be essential for the ATPase activity of SecA in preprotein translocation. Transcription analysis indicates that *R. rickettsii secA* is expressed monocistronically from the canonical prokaryotic promoter with a transcriptional start point located 32 nucleotides upstream of the *secA* initiation codon. Complementation analysis shows that the full length SecA protein from *R. rickettsii* and *R. typhi* fails to restore growth of *E. coli* strain MM52 *secA51* (ts) at non-permissive temperature (42^{o}C) despite the detection of SecA protein expression by Western blotting. However, the chimeric SecA protein carrying N-terminal 408 amino acids of *R. rickettsii* SecA fused with C-terminal 480 amino acids of *E. coli* SecA restores the growth of *E. coli* strain MM52 *secA51* (ts) at the non-permissive temperature (42^{o}C). These results suggest the amino-terminal ATPase domain is highly conserved, while, the C-terminal domain appears to be species-specific.

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FeoB, ferrous iron transporter of *Francisella tularensis*

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Francisella tularensis is a gram-negative bacterium that causes tularemia, a potentially deadly zoonotic disease. Spread of the disease is usually by contact with infected animals, but it can also be acquired by inhaling aerosolized bacteria. In the mammalian host, the bacterium is an intracellular pathogen, residing in membrane-bound phagosomes early in infection. Iron, a scarce but essential nutrient for many invading pathogens, is also important for proliferation of *F. tularensis* within macrophages, but iron acquisition mechanisms in this organism remain uncharacterized. Using BLAST analysis, we have identified *FtfeoB*, a homolog of the ferrous iron transporter gene *feoB* in the Type A virulent SchuS4 genomic sequence database (http://artedi.ebc.uu.se/Projects/Francisella/) as well as in the genomic sequence of the attenuated Type B live vaccine strain (LVS) (http://bbrp.llnl.gov/bbrp/bin/f.tularensis_blast). *ftfeoB* is preceded by an enteric *fur*box –like sequence, suggesting that expression of this gene is regulated in response to iron levels. We have used semi-quantiitative RT-PCR analysis to show that the *ftfeoB* transcript is induced under iron-limiting conditions. We have cloned the *feoB* genes from two strains of *F. tularensis*, the vaccine strain LVS and a Type A virulent strain TI0902, using primers based on the genomic sequence of the SchuS4 strain. The TI strain *feoB* sequence is identical to SchuS4, whereas the LVS gene only displays a small number of sequence differences. We have expressed *his6*-tagged *ftfeoB* genes under arabinose induction control in *E. coli* and are testing for *feoB* function by complementation and transport assays. In order to determine the role of *ftfeoB* in *F. tularensis*, we are working towards generating a null mutant in the LVS strain by replacing the *feoB* gene with a kanamycin-resistance marker.

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Identification of a Novel Protein (PA0122) from *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an important opportunistic pathogen causing chronic lung infections in cystic fibrosis (CF) and immune compromised patients. *P. aeruginosa* strains that initially infect CF patients usually have phenotypes similar to those in the environment. However, compared to these early isolates, strains from chronic infections have unique phenotypic properties including the over production of the polysaccharide alginate, decreased production of exoproducts, decreased expression of lipopolysaccharide O antigen, and an altered lipid A. It is likely these chronically colonizing strains have alterations in expression of other factors. Here we identify genes that show differential expression; these studies may lead to novel targets for vaccine or drug development, new diagnostic reagents, as well as further our understanding of *P. aeruginosa* pathogenesis.

In this study, *P. aeruginosa* GeneChip Microarray analysis was used to monitor the global gene expression profile in two genetically similar, but phenotypically distinct strains of *P. aeruginosa*: 383 (non-mucoid) and 2192 (mucoid), which were isolated from a single CF patient. Strains were grown in Luria broth at 37°C with shaking to reach mid-log phase (optical density of 0.5 at 600 nm). Based on microarray analysis, several genes were identified that showed differential patterns of expression between these two strains. Among the genes whose expression changed significantly was PA0122. This gene showed a higher expression level (8.02 fold) in 383 compared with 2192. The PA0122 gene is predicted to encode a 136-amino acid "conserved hypothetical protein" with a molecular size of 15 kDa, and p*I* of 4.65. PA0122 protein had limited similarity with known proteins from the databank, but is homologous to the fungal *Aspergillus fumigatus* (Asp)-hemolysin protein and has a motif that matched with the fungal Aegerolysin family of proteins. Protein prediction revealed a 21 amino acids transmembrane region at the N-terminus as well as predicted cleavage site at the C-terminus with short stretch of hydrophobic amino acids. This gene has been cloned from strain 383, and expressed as PA0122-His tagged protein in *Escherichia coli*. This recombinant protein was used for the polyclonal antibody production in mice, and the antibody was used to detect the presence of PA0122 protein in *P. aeruginosa*. Western blotting of 383 revealed PA0122 in the supernatant and as well as in the cell lysate; protein levels were dependent on growth phase and cell density. Using 2-D Western blotting, PA0122 protein has been localized at the similar mass of 16 kDa and p*I* of 4.5, as it was predicted earlier in 383, confirming that PA0122, which had only be recognized by an opening reading frame, encodes a novel protein in *P. aeruginosa.* The presence of PA0122 protein varied among other *P. aeruginosa* isolates including both non-mucoid and mucoid strains. Interestingly, PA103 showed a different protein pattern compared with other strains. Real time-PCR characterization of PA0122 revealed that the gene expression levels correlate with the observed protein expression pattern in 383.

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Identification of DinB, a novel DNA polymerase of *Pseudomonas aeruginosa*.

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Recent work has shown that genetic variation may play an important role in the pathogenicity of *Pseudomonas aeruginosa*; examples include alginate over-production by mucoid *P. aeruginosa* in the lungs of cystic fibrosis patients, colony morphology variants arising in biofilms, antibiotic resistant and morphology variants during long-term antibiotic treatment, and isolates with a hypermutable phenotype from the CF lung. In an effort to gain insight into the mechanisms underlying *P. aeruginosa* genotypic variation, we have initiated studies of the DNA repair and replication machinery of *P. aeruginosa*. In *E. coli*, the error-prone DNA polymerase DinB (Pol IV) is induced following DNA damage. DinB, which is regulated by the SOS response, has low processivity and low fidelity when copying across specific sequences. It has been hypothesized that DinB is involved in adaptive mutation of bacterial populations. *P. aeruginosa* has a previously uncharacterized DinB orthologue (PA0923) that is 49% identical to *E. coli* DinB. The goal of this work is to determine the biochemical and biological role of *P. aeruginosa* DinB and its potential link to genetic variation. We have cloned the gene encoding DinB from *P. aeruginosa* PAO1 and generated purified DinB protein. We have also developed an *in vitro* DNA replication assay using extension of a radioactively labeled DNA primer as a measure of DNA polymerase activity. With this assay, we have shown that *P. aeruginosa* DinB indeed has DNA polymerase activity. This protein also appears to be error-prone. Using *lac*+ reversion assays, we discovered that over-expression of DinB causes a higher mutation frequency across specific DNA sequences when compared with cells lacking DinB. Based on the presence of a putative LexA-binding site upstream of *dinB*, we hypothesized that *dinB* is under SOS control and LexA-mediated repression. To test this, cultures of *P. aeruginosa* were treated with the DNA damaging agent mitomycin C. When extracts from these cultures were used in DNA binding assays, we observed decreased binding of a protein(s) in the cells treated with mitomycin C. This suggested that DNA damage may serve as a signal for release of a repressor, potentially LexA, from *dinB*. In support of this, DNA binding activity was observed in extracts derived from wild type but not *lexA*-deficient *P. aeruginosa*. Taken together, these data indicate that *P. aeruginosa* DinB is a DNA damage-inducible DNA polymerase, with an error-prone mechanism of DNA synthesis. The induction of such a mutation-prone polymerase could play a role in the virulence of *P. aeruginosa*, especially in conditions of genotypic variation like the CF lung or in the biofilm mode of growth.

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Characterization and Analysis of a Two-Component Regulatory System In *Borrelia burgdorferi*

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Lyme disease, caused by *Borrelia burgdorferi*, is the leading vector-borne disease in North America and Europe. The ability of B. *burgdorferi* to survive in different environments within different hosts indicates that it is able to rapidly adapt to changing environmental conditions. In this study we have begun the characterization of a putative two-component regulatory system made up of Bb0419 (the response regulator) and Bb0420 (a sensory transduction histidine kinase). First, PCR analyses were performed to determine if this gene cluster is universally present in Lyme disease isolates as would be expected if it plays a central role in virulence or environmental adaptation. The gene cluster was detected in all isolates tested and polymorphisms were not evident. The genetic conservation of BbO419 was further demonstrated through the sequencing of Bb0419 from a diverse group of isolates. The close spacing between Bb0419 and BB0420 suggested that these genes were co-transcribed. This was confirmed through reverse transcriptase PCR. Using real time PCR, we also demonstrated that transcription of the operon was not influenced by cultivation temperature. To identify the sub-cellular localization of Bb0419, *Borrelia burgdorferi* cells were subjected to Triton-X-114 extraction and phase partitioning. Bb0419 partitioned exclusively to the detergent insoluble phase, thereby indicating that Bb0419 is a cytoplasmic protein. To assess the influence of Bb0419 on global protein expression profiles, a knockout mutant of Bb0419 has been generated through allelic exchange mutagenesis. Initial efforts are underway to characterize this mutant. Collectively, these analyses represent the starting point for the characterization of a two-component regulatory system in *Borrelia burgdorferi*.

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Transcriptional profiles of innate immunity knockout mice

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Extracellular pathogens are detected primarily by the family of Toll-like receptors (TLR), which are sentinels of innate immunity that recognize bacterial pathogen associated molecular patterns. However, many clinically significant pathogens spend all or part of their life cycle within the cytoplasm of the host cell. Recently, it has been shown that host cells detect the intracellular pathogen *Listeria monocytogenes* in the cytoplasm via a novel and poorly characterized Cytoplasmic Surveillance Pathway (CSP). Even though the CSP is independent of TLR-mediated signaling, it intersects with the TLR-mediated MyD88 independent pathway leading to the production of Interferon Beta (IFN-β). Therefore, the transcriptional host-response to infection with intracellular bacterial pathogens depends on a combination of TLR-dependent and TLR-independent mechanisms. Using a mouse primary macrophage model of infection, the Fawcett lab has previously employed DNA microarrays to describe the overall host transcriptional responses. Now, using a panel of knockout mice defective in various aspects of innate immunity signaling, we have begun the process of "peeling the onion" of this multi-layered and multi-staged response. As before, we employ *Listeria monocytogenes* as a model stimulus of the CSP, and infect mouse bone marrow derived macrophages obtained from knock-out mice. To asses the relative contributions of the different signaling pathways to the overall response we are now using a new generation of mouse whole-genome spotted 70-mer oligo microarrays to analyze the host global transcriptional responses to *Lm* infection. This transcriptional analysis is being complemented by using ELISA to monitor the production of specific protein products such as TNF-α and IFN-β.

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Caenorhabditis elegans - A Novel Host for *Candida* Infection

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We have an ongoing interest in using simple, invertebrate organisms as model hosts for the study of human pathogens. If both the host and pathogen can be genetically manipulated, these model systems can be used in large-scale genetic studies to concurrently identify microbial virulence-related genes and host innate immune system determinants. In this study, we describe a novel system for studying the molecular and genetic mechanisms of *Candida* pathogenesis using the nematode model organism *Caenorhabditis elegans*. Many pathogenic *Candida* species, including *C. albicans* and *C. glabrata*, cause lethal infections in nematodes. Adult nematodes die within 48 hours while feeding on lawns of live *Candida* but live 2-3 weeks on amphotericin-killed yeast. Nematode killing is correlated with the accumulation of yeast within the worm alimentary tract and is dependent on the sex and developmental stage of the worm. *C. albicans* mutants *efg1*/*efg1* and *efg1*/*efg1 cph1*/*cph1*, known to be attenuated in systemic infections in mice, are less virulent in *C. elegans* killing. Although these mutants have been shown to be defective in yeast-to-hyphal transition, there is no evidence of filamentation within the nematode alimentary tract, suggesting that the Efg1 and Cph1 pathways may regulate other factors important for nematode killing. Similarly, the *C. albicans* isocitrate lyase mutant *icl1*/*icl1*, previously shown to be attenuated in a murine infection model, is also virulence-defective in worms. These results suggest that at least some aspects of *Candida* pathogenicity may be conserved between evolutionarily distant hosts. By exploiting the fact the *C. glabrata* has an obligate haploid genome that can be randomly mutagenized using the nonreplicative yeast vector YIplac211, we have used *C. elegans* to screen a library of 1028 *C. glabrata* mutants and identified 14 clones that are attenuated in nematode killing. The loci disrupted in 12 of these mutants have been identified within or adjacent to genes involved in a variety of cellular processes, including endocytosis, protein processing, fatty acid metabolism, DNA repair, chromatin silencing, and transcriptional activation. One mutant contains an insertion in the kexin-like endoproteinase gene *CgKEX2*, one of only two *C. glabrata* genes to have ever been studied in the context of virulence. The insertion site of five mutants are within the promoter region of genes known to be essential for in vitro growth of *Saccharomyces cerevisiae* and/or *C. albicans*. We speculate that *C. elegans* may be a useful model for the identification of novel *Candida* genes important in mammalian pathogenesis and for continuing exploration of host response to infection.

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Interleukin-12 is required for the innate host defense against lung colonization by *Pseudomonas aeruginosa.*

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Respiratory infection with *P. aeruginosa* is a prelude to fatal pneumonia in cystic fibrosis (CF). While CF knockout (ko) mice show some similarities to the pathogenesis of the disease in humans, the lung pathology is missing from these mice, indicating that genetic modifiers exist that can increase the inherent resistance level to infection. Using an aerosol infection mouse model, we previously found that the inbred mouse strains can be generally classified into two groups based on lung colonization and mortality: susceptible vs. resistant mice. While DBA/2 mice are susceptible to infection, 129/SV, A/J, BALB/c, C57BL/6, C3H/HeN and FVB mice are not. Furthermore, colonization with *P. aeruginosa* causes a strong induction of interleukin-12 (IL-12) in the resistant but not in DBA/2 mice. IL-12 and tumor necrosis factor-α (TNF-α) co-stimulate the release of interferon-γ (IFN-γ) from natural killer and T lymphocytes, which in turn activates the bactericidal mechanisms in macrophages. While it is clear that IL-12 is of importance to the host defense against intracellular bacteria, little is known about whether IL-12 is needed for lung protection from extracellular pathogens such as *P. aeruginosa*. To dissect the function of this cytokine in lung infection, IL-12p40 ko mice (BALB/c-IL12b^{tm1Jm}) were tested for colonization and mortality in this model. When given the same dose of bacteria as measured in colony forming units immediately after exposure, the IL-12 ko mice, like DBA/2, had a significant increase in viable counts recovered from the lungs at the 6 h time point as compared to the BALB/c or C57BL/6 mice. Within 48 h after infection, 100% mortality was observed in the IL-12 ko and DBA/2 strains, whereas no mortality was seen in the BALB/c or C57BL/6 strains. Intraperitoneal (i.p.) injections of recombinant mouse IL-12p40 homodimer 2 h before and 2 h post infection at a dose of 0.6 µg per mouse cured 67% and 47% of DBA/2, respectively, indicating a protective role for this cytokine. However, no protection was observed in DBA/2 mice with an increased dose of 1.0 µg per mouse. Taken together, IL-12 is required for the innate defense against extracellular bacterial lung infections. IL-12-deficient mice are unable to effectively clear respiratory colonization causing mortality from the *P. aeruginosa* infection. Absence of IL-12 in the resistant mice causes the same phenotypic expression of the susceptibility trait as seen in DBA/2 mice. Treatment of DBA/2 mice with IL-12 during infection with *P. aeruginosa* shows a dose-dependent protection in these susceptible animals. Coupled with two previous studies that show a critical role of TNF-α in the *P. aeruginosa* infection and that IL-12p40 is expressed only in lymphoid cells, our results suggest that IFNγ-dependent activation of alveolar macrophages may be a key portal in the fight against lower respiratory tract colonization by *P. aeruginosa*. A better understanding of the innate lung defense network in mice will lead to the development of novel therapeutics for CF.

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Role of *Borrelia burgdorferi* aggregates in the expression of outer surface protein C (OspC)

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Borrelia burgdorferi is the tick borne spirochete responsible for Lyme disease. During tick transmission, *Borrelia* alters its gene expression profile to help it survive in the new host. The *ospC* gene coding for outer surface protein C (OspC) is induced during tick feeding and OspC is thought to play a role in the transmission and subsequent infection of mammals. In the feeding tick gut precise signals for OspC induction are not defined, however *in vitro* studies suggest that high temperature, high cell density, low pH and low oxygen tension cause OspC induction. It is still obscure how these environmental factors are interrelated and contribute to differential gene expression within ticks. We observed that *Borrelia* cells aggregate during *in vitro* growth at high temperature and high cell density. This encouraged us to investigate the role of *Borrelia* aggregates in OspC expression. When aggregates and free cells were sorted by fluorescence assisted cell sorting (FACS), 15% of free cells and more than 90% of aggregates expressed OspC. Our western blot analysis confirmed that aggregates contained more OspC than free cells. To understand the biological relevance of our *in vitro* studies, we observed the *Borrelia* in feeding ticks. Bacteria within feeding ticks also aggregated and OspC was enriched in aggregates. We propose that in the feeding tick gut, *Borrelia* multiplies rapidly to high numbers while being exposed to high temperature which leads to aggregation, which is the key signal for the induction of OspC within feeding tick.

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The Effects of Insertional Inactivation of Response Regulator Genes on Hyaluronate Lyase and Other Virulence Factors of *Streptococcus pyogenes*

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Streptococcus pyogenes, or Group A Streptococcus, has only one host and that is man. This gram-positive bacterium has become exquisitely adapted to a variety of host environments and is able to evade host defenses and persist, in part, because of the coordinated expression of the numerous virulence factors it employs. Analysis of published genomes of GAS has identified 13 two-component signal transduction systems that are potential environmental regulators. Six of these, *fas*BCA, *cov*RS/*csr*RS, *syc*FG, *srt*RK, *sal*RS and *irr/ihk* have similarity to other regulatory systems. Mutagenic plasmid constructs containing a portion of 12 of these regulator genes (Ribardo et al, 2004, Infect. Immun.72: 3668-3673) will be used to create insertional inactivations in *S. pyogenes* M-4 strain 635-02, a hyaluronate lyase producer. One of these regulators (*sycFG*) is essential to the cell so is not included in these studies. PCR analysis suggests the M4 strain contains homologs of the remaining12 response regulators, while the M-type 22 strain 10403 appears to lack *srt*RK. The plasmids were electroporated into the parent strain and the resulting electrotransformants were screened for loss of activity of the lyase activity as well as other virulence factors. Inactivation of SPT-9 and SPT-12 show no effect on hyaluronate lyase activity. This suggests that neither of these regulatory genes is responsible for the control of this enzyme. Inactivation of the remaining regulators is currently underway. As mutants with inactivated regulators are obtained, other virulence factors including hemolysin and the cysteine protease (SpeB) will also be screened to see if they are affected.

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Caenorhabditis elegans as a model for *Yersinia pestis* infection

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Caenorhabditis elegans was recently developed as a model for host-pathogen interactions. Many of the same bacterial virulence factors important for pathogenicity in mammalian systems were also shown to be required for killing of *C. elegans*. Previous work has been done to study *Yersinia pestis*, a Gram-negative bacterium and the causative agent of plague, using *C. elegans* as a model organism. *Y. pestis* strain Kim6+ has been shown to kill *C. elegans* in a biofilm dependent manner. It is suggested that the biofilm hinders nematode movement and causes death through starvation. In these studies, other strains of *Y .pestis* exhibited a weaker lethality which was independent of biofilm formation. In order to identify this alternative mechanism of virulence, we tested various *Y. pestis* strains mutant in various virulence loci. We selected for our experiments the Kim5 strain, which lacks the *pgm* locus but contains all three *Y. pestis* virulence plasmids. Similar to *S. enterica* infections, *Y. pestis* Kim5 caused a persistent colonization in the gut of the nematode. This provides a system for a high-throughput forward genetic screen for *Y. pestis* virulence factors independent of biofilm formation. A transposon insertion library of *Y. pestis* was prepared and 984 transposon insertion *Y. pestis* mutants were screened to identify strains exhibiting reduced virulence in *C. elegans*. A preliminary screen identified 143 mutants exhibiting reduced killing. In a secondary screen of the identified mutants, the number of nematodes used was increased and the entire course of infection was followed. The rescreen confirmed the reduced virulence in *C. elegans* for 28 mutants (2.8% yield). The results obtained are consistent with a previous study of *S. enterica* mutants using *C. elegans* as a host system. We are in the process of characterizing these mutants. Our experiments demonstrated that *Y. pestis* Kim5 causes a lethal internal infection of *C. elegans*, creating a useful model to study *Y. pestis* pathogenesis.

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Identification of the immunodominant epitopes of an outer surface protein family of the Lyme disease spirochetes

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B. burgdorferi, *B. garinii* and *B. afzelii* are the causative agents of Lyme disease. The *Borrelia* are atypical in that a large portion of their genome is in the form of plasmids that are both linear and circular in structure. Plasmids comprise nearly 50% of the total genetic material. A significant portion of the plasmid carried genes encode outer surface proteins that form extensive paralogous protein families. Surface antigens likely play a key role in defining the host-pathogen interaction. This report is focused on an assessment of the humoral immune response to proteins belonging to family 163. Family 163 contains 5 members (BBO40, BBP39, BBL40, BBN39 and BBQ47). BBP39 and BBL40 are identical in sequence and are henceforth referred to as BBP39. These proteins are surface exposed and antigenic during infection. In this report we have cloned and expressed BBP39, BBO40, BBQ47 and BBN39 as S-Tag fusion proteins and used these recombinant proteins to monitor the temporal nature of the antibody response to these antigens during experimental infection of mice with various isolates of *B. burgdorferi*. The immunodominant regions of each protein have also been identified through immunoblot analyses of a series of N-terminal and C-terminal truncations of each protein. These analyses have led to the delineation of the targets of the antibody response during infection and of the specificity of the antibody response to the family 163 proteins. It is particularly worth noting that a very strong IgG response was detected for P39 just two weeks into infection indicating that this protein might play an important role in the establishment of early stages of infection. RT-PCR analyses were also performed to determine if family 163 genes are co-transcribed with other flanking genes. Most of these genes were found to be expressed as two gene operons. Collectively the analyses presented here enhance our understanding of the host-pathogen interaction, differential gene expression by *B. burgdorferi* and suggest that some members of family 163 paralogs play an important role during early infection.

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Microarray Analysis of the Host Defense Response of *Caenorhabditis elegans* to *Salmonella enterica* serovar Typhimurium

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The first response of the immune system is innate immunity. Our laboratory along with others has shown that *C. elegans* has conserved mammalian components of innate immunity and can serve as a genetic model to study host-pathogen interactions. Studying the mechanisms by which the human pathogenic bacterium, *S. enterica*, modulates defense responses at the transcriptional level should provide not only insight into how it causes disease but also help to reveal the functions of the components of innate immunity. Microarray analysis measuring the gene expression of nematodes challenged with *S. enterica* compared to *E. coli* detected over 342 genes with at least 2-fold increased expression and 334 genes with at least 2-fold decreased expression. Genes with altered levels of expression encoding carboxylases, a bactericidal permeability-like protein, G-coupled receptors, lectins, lipases, map kinases, mucins, a peroxidase, proteases, transmembrane proteins, and transcriptional regulators were identified.
The *C. elegans* genes responsive to *S. enterica* were examined to identify human homologues and 181 genes were found. We have further characterized these phylogenetically conserved genes from the microarray analysis for their potential role in *C. elegans* host defense. RNA-mediated interference was used to inhibit the gene function of these 181 genes in *C. elegans* followed by challenge with *S. enterica*. The susceptibility of the RNAi nematodes to *S. enterica* was evaluated by determining worm mortality percentages on day 3, day 4, and day 6 post-exposure and 47 *ess* (enhanced susceptibility to *S. enterica*) genes and 24 *ers* (enhanced resistance to *S. enterica*) genes were identified. We are currently re-screening these genes to validate their role in *C. elegans* host defense against *S. enterica* as well as other human pathogens. Further studies will be performed to elucidate the pathways and interacting complexes associated with these genes that are essential for conserved host defense responses.

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Monocytes and macrophages are important for host defense against *Clostridium perfringens* gas gangrene infections.

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Clostridium perfringens is the most common cause of gas gangrene (clostridial myonecrosis). In response to infection, polymorphonuclear leukocytes (PMNs) and monocytes are recruited to the tissues by the animal host. We examined the kinetics of leukocyte recruitment to the site of a gas gangrene infection. We injected 10^9 bacteria/mouse (a lethal dose which would cause gas gangrene) and 10^6 bacteria (a non-lethal dose, no gangrene) into the hind leg muscle of Balb/c mice. At 10, 60, 120 and 180 minutes post infection, the mice were euthanized and the infected leg muscles removed and serial sections of tissue were placed on microscope slides. The sections were stained by H/E and examined for the presence of PMNs and monocytic cells at the site of infection. There was a statistically significant lower level of phagocytes in the mice injected with 10^9 cells than those injected with 10^6 bacteria over most of the time points examined. Using human PMNs and monocytes in in vitro assays, we found that these cells can efficiently phagocytose and kill *C. perfringens* in the presence of complement under both aerobic and anaerobic conditions. To determine the relative importance of PMNs and monocytes at limiting a *C. perfringens* gas gangrene infection, we depleted these cells in Balb/c mice: PMNs with an intraperitoneal injection of the monoclonal antibody RB6-8C5 and vascular monocytes with clodronate-filled liposomes. The mice were infected one day after PMN or monocyte depletion with 10^9, 10^8, 10^7, or 10^6 cfu/mouse. For the PMN depleted mice, there was no statistically significant difference between control (PBS injected) and experimental mouse survival. For the monocyte depleted mice, a statistically significant decrease in mouse survival was seen in the mice infected with 10^8cfu/mouse by 24 hours post infection. Together these results suggest that while large numbers of *C. perfringens* can keep the level of all phagocytes low in the vicinity of the bacteria, monocytes play a more important role than PMNs in combating the development of a *C. perfringens* gas gangrene infection.

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Analysis of the Molecular Basis of Dissemination Characteristics of Clinical Lyme Disease Isolates

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Lyme disease is a tick borne zoonosis caused by the spirochetes *Borrelia burgdorferi*, *B. garinii* and *B. afzelii*. There is considerable variation in the clinical presentation of Lyme disease during both its early and late stages. A correlation appears to exist between specific genospecies and specific clinical manifestations. *B. burgdorferi* can be classified into three major genotypes based on a combination of PCR amplification and restriction analysis of the ribosomal spacer between the 16S and 23Ss rRNA genes. The three genotypes are referred to as RST-1 (ribosomal spacer type), RST-2 and RST-3. Evidence suggests that RST-1 type *B. burgdorferi* disseminates more efficiently than other genotypes and can specifically elicit the formation of multiple erythema migrans lesions. One potentially contributing factor that could influence dissemination capability is the ability/inability of the spirochetes to bind the complement regulatory proteins, factor H and FHL-1. These proteins are key regulators of the alternative complement pathway. *B. burgdorferi* produces two major factor H binding proteins, OspE and BBAA68. The binding of factor H to the cell surface may facilitate enhanced cleavage of C3b and thereby decrease the efficiency of opsonophagocytosis. The goals of this study were to compare the properties of the factor H binding proteins produced by different RST types and determine if differences exist in overall factor H binding ability. To determine if OspE and BBA68 are produced by isolates of each RST type, immunoblot analyses were performed using anti-ospE and anti-BBA68 antisera. The ability of each isolate to bind factor H was directly tested using the factor H affinity ligand binding immunoblot assay. These approaches revealed significant differences in the properties of the factor H binding proteins. To identify the molecular basis of these differences, the *ospE* gene was amplified from several strains, sequenced and evolutionary analyses were conducted. These analyses revealed that *ospE* is highly conserved among RST1 isolates but variable among RST3 isolates. Studies are underway to quantitatively assess potential differences in the ability of the factor H binding proteins from different RST types to bind factor H.

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The Role of Carbohydrates in Regulating Type IV Pili-Mediated Motility in *Clostridium perfringens*.

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Clostridium perfringens is a gram-positive anaerobic pathogen that can cause a range of diseases, ranging from enteric diseases, such as acute food poisoning, to tissue infections, such as gas gangrene. A physiological trait that has never been observed in *C. perfringens* is motility. We observed that bacteria on solid media behaved in a manner consistent with type IV pili-mediated motility. The genome sequences of three strains of *C. perfringens* (ATCC 13124, SM101 and strain 13) have been determined, and motility has been observed in all three strains. Examination of the annotation revealed the presence of type IV pilus (TFP) related genes in three locations. A series of tests were conducted to attempt to identify factors controlling *C. perfringens* motility. The glucose concentration in our standard growth medium is 20 grams per liter, and at this concentration motility was not observed for 48 hours; but colonies growing on lower concentrations of glucose exhibited motility in shorter periods of time. Media containing 1% glycerol inhibited motility by all strains. Motility was observed on agar concentrations up to 3%, which is very high for bacterial twitching motility on a solid surface. A natural variant of strain 13, designated SM123, was isolated after exposure to J77-4 macrophages. SM123 appeared to be altered in the timing of its motility since motility was observed on plates within 4-6 hours after plating, compared to its parent strain, which typically requires 18 hours for motility to be visible. Exopolysaccharide (EPS) assays have indicated that SM123 posses nearly 5 times the EPS levels as strain 13. These characteristics suggest that carbohydrate metabolism plays a regulatory role in initiating movement and that synthesis of the polysaccharide capsule may be important in facilitating movement across solid surfaces.

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Endonuclease I Significantly Impairs Bacteria-Mediated Transfection of Eukaryotic Cells.

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Enteroinvasive bacteria represent an alternate, needle-free mode of delivery for the administration of DNA vaccines. Numerous groups have investigated a number of different species of enteroinvasive bacteria as delivery vectors, with varying results. We were interested in optimizing our *Shigella flexneri* vaccine candidate CVD 1208 as a DNA vaccine delivery vector. CVD 1208 has successfully completed a phase 1 clinical trial and has been used as vector for the delivery of a DNA vaccine encoding measles virus hemagglutinin in cotton rats (J. Virol, 77:5209-17). Herein we show that a gene encoding periplasmic endonuclease I (*endA*) derived from CVD 1208, when used to reconstitute endonuclease I activity in invasive DH5α, significantly impairs bacteria-mediated transfection of BHK21 cells. Correspondingly, removal of *endA* from the chromosome of CVD 1208 significantly improved its ability to transfect BHK21 cells. ia-mediated transfection of a eukaryotic cell.

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The Regulation of Quinolone Signaling in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that causes chronic lung infections in cystic fibrosis patients and is a major source of nosocomial infections. This bacterium controls many of its virulence factors using its two quorum sensing systems, *las* and *rhl*. The *las* system is composed of the LasR regulator protein and its cell to cell signal, 3-oxo-C_{12}-HSL, and the *rhl* system is composed of RhlR and its cell to cell signal, C_4-HSL. A third molecule, the *Pseudomonas* quinolone signal (PQS; 2 –heptyl-3-hydroxy-4-quinolone), has also been shown to regulate numerous virulence factors. We have recently been studying an operon required for PQS synthesis, *pqsABCDE*. Transcription of *pqsA*, and therefore PQS production, is positively regulated by the *las* quorum sensing system and negatively regulated by the *rhl* quorum sensing system. PqsR is a LysR-type transcriptional regulator protein that positively controls *pqsA* transcription, as well as the anthranilate synthase genes, *phnAB*. Using DNA mobility shift assays and β-galactosidase assays, we have studied the regulation of *pqsR* and its relationship to *pqsA*, *lasR*, and *rhlR*. We show here that PqsR binds the promoter of *pqsA*, and that this binding increases dramatically in the presence of PQS, suggesting that PQS may be the coinducer molecule for PqsR. We have mapped the transcriptional start site for *pqsR* and found that transcription of *pqsR* is positively regulated by *lasR* and negatively regulated by *rhlR*. We also show that PqsR expression can restore PQS synthesis in a *lasR* mutant. These results suggest that a regulatory chain occurs where *pqsR* is under the control of *lasR* and *rhlR*, and *pqsR* in turn controls *pqsA*, which is required for the production of PQS.

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Investigating the Translocation of Pertussis Toxin from the ER to the Cytosol – Evidence for Exploitation of the ERAD Pathway

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Bordetella pertussis produces an exotoxin known as pertussis toxin, an important virulence factor. PT holotoxin comprises one A subunit (S1), bearing the enzymatic activity, associated with a pentameric ring of B subunits. PT is an ADP-ribosyltransferase that modifies several mammalian heterotrimeric G proteins. Several bacterial toxins including cholera toxin and also the plant toxin ricin are believed to undergo retrograde intracellular transport through the Golgi apparatus to the endoplasmic reticulum. The ERAD pathway involves removal of misfolded proteins from the ER and degradation (after return to the cytosol); this is a candidate pathway for exploitation by S1 and other toxins for ER to cytosol transport. In the cytosol ERAD substrate proteins are ubiquitinated at lysine residues, for targeting proteins to the proteasome for degradation. For utilization of this pathway PT must presumably avoid ubiquitination and degradation. We hypothesize that since S1 lacks lysine residues it is credible that ubiquitination may be avoided. We predict that PT toxicity will be reduced by the addition of lysine residues allowing ubiquitination and degradation to occur. Mutant forms of PT were engineered replacing one, two or three arginines with lysine residues, in a variety of locations on S1. Several mutants (with multiple lysine substitutions) have been identified with wildtype *in vitro* enzymatic activity but reduced cellular activity, consistent with our hypothesis. Previous studies have provided evidence that trafficking of PT from the ER as part of the cellular intoxication pathway occurs. CHO cells were stably transfected with constructs containing S1 alone or coupled to a signal peptide that targeted S1 to the ER. Even when directed to the ER, S1 ADP-ribosylated target cytosolic G proteins. However S1 expression levels were significantly greater than cell-associated levels from exogenous PT treatment. To investigate *in vivo* expression and transport of S1 bearing mutations, constructs were engineered with S1 under control of a doxycycline-inducible promoter. S1 expression was controlled in a dose dependant manner in CHO cell transfectants; however, low background expression (uninduced) was observed. This uninduced expression was sufficient to bring about complete ADP-ribosylation, even at levels undetectable by western blot. The addition of the signal peptide had no effect on PT's ability to ADP-ribosylate target G-proteins. From these data we can conclude that PT is able to translocate from the ER to the cytosol, and that very low numbers of S1 molecules are sufficient for full activity. Similar S1 constructs were engineered with single and triple lysine mutations under the inducible promoter in the presence and absence of the signal peptide. These constructs have been transfected into CHO cells and the expression and intracellular activity of these construct is being determined.

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Demonstration of co-transcription of a 28-gene operon from a 32kb circular plasmid of *Borrelia burgdorferi* that is induced by the DNA alkylating agent, MNNG: Additional evidence for the existence of bacteriophage in *Borrelia burgdorferi.*

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Species of the genus *Borrelia* possess a unique genome that is comprised of a linear chromosome and a series of linear and circular plasmids. Most isolates carry multiple circular plasmids of 32kb that have been designated as the cp32s. These plasmids carry several genes that are environmentally regulated and that encode putative virulence factors including the factor H binding proteins of the OspE protein family. There is increasing evidence that the cp32s are bacteriophage and hence could prove to be very important in the lateral transfer of genes encoding virulence factors. In this report the transcriptional expression patterns of 28 genes thought to comprise a "phage transcriptional unit" were determined. Using reverse transcriptase PCR we demonstrate that orfs BBL01 through BBL28 can be transcribed as a monocistronic mRNA. Realtime RT-PCR further demonstrated that transcription of these genes is inducible by the DNA alkylating agent MNNG. The level of detectable transcript from these genes increased by 2500 fold upon MNNG induction. Genes outside of the operon were found not induced by MNNG. Recombinant protein was generated for several of the genes contained in the operon in order to generate important reagents for future analyses and to determine if these proteins elicit an Ab response during infection. Infection serum obtained from a mouse infected with *B. burgdorferi* B31 MI did not react with the recombinant proteins demonstrating that these proteins are not antigenic during infection. It remains to be determined if these genes are upregulated during infection. In summary, the data presented here provide evidence supporting the contention that the cp32s are prophage that may play an important role in *Borrelia* pathogenesis.

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The Sigma Factor AlgT Inhibits *Pseudomonas Aeruginosa* Flagellum Biosynthesis By Repressing Expression Of The Flagellar Regulator FleQ

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Pseudomonas aeruginosa is the terminal pathogen in individuals suffering from cystic fibrosis (CF). Initially, environmental strains colonizing the CF lung generally have a non-mucoid, motile phenotype. However, as the disease progresses, many of these strains convert to a mucoid and/or non-motile phenotype, thus providing the microorganism with a selective advantage in the CF lung. We observed that in CF isolates, the mucoid and the non-motile phenotype occur predominantly, though not exclusively, together. Studies in our laboratory revealed that alginate and flagellum biosynthesis are inversely regulated by the alternative sigma factor AlgT (AlgU, σ^{22}). The goals of the current study are to identify the highest target of AlgT within the intricate flagellar hierarchy and to determine the AlgT-mediated regulatory mechanism involved in the inhibition of flagellum biosynthesis. Microarray analysis comparing mRNA levels of isogenic AlgT+ and AlgT- *P. aeruginosa* was performed to determine which genes within the flagellar biosynthetic pathway are controlled by AlgT. The results showed that the vast majority of flagellar genes were significantly downregulated in the presence of AlgT. A pronounced inhibitory AlgT-effect was observed in several genes essential for proper flagellum expression, including the gene coding for the flagellar master regulator, *fleQ*. Promoter fusion assays in isogenic AlgT+ and AlgT- strains confirmed this negative effect of AlgT on the transcription of *fleQ*. In addition, motility assays and TEM showed that overexpressing FleQ in mucoid, non-motile CF isolates restores both flagellum biosynthesis and motility. Electrophoretic mobility shift studies using purified AlgT and extracts derived from isogenic AlgT+ and AlgT- strains revealed that AlgT inhibits *fleQ* transcription in an indirect manner. Together, these data clearly show that AlgT mediates the negative control of the flagellar biosynthetic by indirectly inhibiting the expression of the master regulator *fleQ*.

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Molecular Regulation of the Csr System in *Salmonella typhimurium* and the Effects on Invasion

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The ability of *Salmonella* enterica serovar Typhimurium (*S. typhimurium*) to penetrate the intestinal epithelium is an essential early step in the pathogenesis of this organism, requiring the expression of invasion genes of *Salmonella* pathogenecity island 1 (SPI-1). We have previously shown that SPI-1 genes are controlled by the Csr system, consisting of the post-transcriptional regulator CsrA and the untranslated RNAs *csrB* and *csrC* that oppose the action of CsrA. Further, we have shown that the Csr system is itself controlled by the two-component regulator BarA/SirA through its regulation of *csrB* and *csrC*. We have also demonstrated using DNA microarrays that CsrA has a broad range of target genes in *S. typhimurium* and that they differ in many cases from those controlled by its ortholog in *E. coli*. To identify CsrA control of *csrB* and *csrC* in *Salmonella*, we examined the levels of each regulatory RNA in response to altered expression of *csrA*. Here we demonstrate by Northern analysis that the loss of *csrA* reduced *csrC* by 2-fold and *csrB* by 2-fold. Additionally, over-expression of *csrA* from an arabinose-inducible promoter increased both *csrC* and *csrB* by 10- fold. Next, we examined the effects on *csrB* and *csrC* caused by the loss of the other. Interestingly, *csrC* was increased 7-fold in a *csrB* mutant, while *csrB* was also increased 7-fold in a *csrC* mutant. These data indicate that each can act to negatively regulate the other. We hypothesized from these findings that CsrA may bind to and stabilize *csrB* and *csrC*. Therefore, we examined the effects of altered CsrA levels on *csrB* and *csrC* using a message stability assay. The production of new RNA was halted with rifampicin and the amount of *csrB* and *csrC* was measured at time points thereafter using reverse transcription PCR. These results showed that the loss of CsrA reduced that stability of *csrC*. *csrC* had a half-life of 62 minutes in the wild type strain, but only 1 minute in the *csrA* mutant. In contrast, the half-life of *csrB* in the CsrA mutant was unchanged from at of the wild type. These results therefore suggest that CsrA directly stabilizes *csrC*, but regulates the levels of *csrB* by an indirect method.

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Rhamnolipid, a *P. aeruginosa*-produced biosurfactant, increases the solubility and bioactivity of the Pseudomonas Quinolone Signal (PQS).

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Pseudomonas aeruginosa is a gram-negative bacterium that is found free-living in the environment and poses a serious threat to human health as an opportunistic pathogen. From a medical perspective, *P. aeruginosa* is well known for its ability to infect immunocompromised individuals as well as those suffering from cystic fibrosis. *P. aeruginosa* is also notorious for its ability to solubilize and degrade a wide array of organic compounds through the production of numerous surfactants and degradative enzymes. The virulence of *P. aeruginosa* is dependent upon cell-to-cell communications. Cell-to-cell signaling allows the bacterium to regulate the expression of virulence factors, such as proteases, phosphatases, lipases, hemolysins, and rhamnolipid, in a highly orchestrated, cell density-dependent manner. One novel *P. aeruginosa* cell-to-cell signal is 2-heptyl-3-hydroxy-4-quinolone, the *Pseudomonas* Quinolone Signal (PQS). While PQS can be found in culture media, the purified signal was relatively insoluble in aqueous solutions. We hypothesized that *P. aeruginosa* produced a compound that increased the solubility of PQS. The study at hand revealed that rhamnolipid, a PQS-controlled surfactant and virulence factor, increased the solubility and bioactivity of PQS. In addition, it was demonstrated that PQS was directly toxic to eukaryotic cells. PQS induced apoptosis in eukaryotic cells and this toxic activity was enhanced in the presence of rhamnolipid. This study is the first to demonstrate that a bacterium produces a compound that will solubilize and thereby augment the bioactivity of a cell-to-cell signal.

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