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The Effects of Low-Shear Modeled Microgravity on *Streptococcus pneumoniae* and Adherent-Invasive *Escherichia coli*

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The Effects of Low-Shear Modeled Microgravity on *Streptococcus pneumoniae* and Adherent-Invasive *Escherichia coli*

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Dedication

To my parents, James and Barbara, and my wife, Amy, whose unending support gave me the strength to persevere.

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The Effects of Low-Shear Modeled Microgravity on *Streptococcus pneumoniae* and Adherent-Invasive *Escherichia coli*

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The effects of low-shear modeled microgravity (LSMMG) were investigated on *Streptococcus pneumoniae* global gene expression and on adherent-invasive *Escherichia coli* (AIEC) physiology and colonization properties. Habitation in space exposes both humans and microbes to microgravity conditions which are characterized by reductions in fluid shear forces. Areas of low-shear stress are also encountered in physiologically relevant regions of the body including the respiratory, gastrointestinal, and urogenital tracts. The LSMMG environment impacts both bacterial physiology and virulence properties and can be modeled using rotating-wall bioreactors known as high-aspect ratio vessels (HARVs).

Previous studies have evaluated the global transcriptional profiles of Gram-negative bacteria; however, no Gram-positive species have been examined. Microarray analysis of *S. pneumoniae* strain TIGR4 (serotype 4), after growth under LSMMG, revealed a dramatic down-shift in gene expression based on cluster analysis. Within this group of responsive genes, statistical analyses revealed that the expression of 81 genes

was significantly altered. These genes were found to be associated with 7 different functional categories, including many which were uncharacterized. Several gene groups shared common functional operons and regulons such as those involved in competence induction, antimicrobial peptide production, and carbohydrate uptake.

While previous studies examining the effects of LSMMG on bacteria have focused on well-characterized strains of both commensal and pathogenic species, there is limited information regarding the effects of LSMMG on clinical isolates associated with Crohn's Disease, an inflammatory bowel pathology. Analysis of wild-type AIEC strain O83:H1 and an isogenic *rpoS* mutant (CAA001), after growth under LSMMG, revealed alterations in environmental stress resistance and increased adherence. Altered resistances to thermal and osmotic stresses were observed by LSMMG-grown AIEC O83:H1, while resistance to oxidative and acid stresses appeared to be *rpoS*-dependent. Further, CAA001 displayed a hyper-adherent phenotype while grown under LSMMG. Tn*phoA* mutagenesis was used to abolish the hyper-adherent phenotype of CAA001 under LSMMG, and the insertion was mapped within the *tnaB* gene, encoding tryptophan permease. Complementation of the *tnaB* gene in the *rpoS tnaB* double-mutant restored adherence capabilities. These findings extend our understanding of how mechanical forces (e.g. LSMMG) can affect the functions of Gram-positive and Gram-negative species.

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List of Abbreviations

AIEC	Adherent-invasive *E. coli*
ANOVA	Analysis of Variance
Ap	Ampicillin
BIP	Bacteriocin-inducing peptide
BLAST	Basic Local Alignment Search Tool
BLP	Bacteriocin-like peptide
CbpA	Choline-binding protein A
CD	Crohn's Disease
CFU	Colony forming units
Cm	Chloramphenicol
CPS	Capsular polysaccharide
CSP	Competence-stimulating peptide
Cy3	cyanine 3 – dUTP
Cy5	cyanine 5 – dUTP

DMEM	Dulbecco's Modified Eagle's medium
ECL	Electrochemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EHEC	Enterohemmorhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FDR	False Detection Ratio
Fur	Ferric uptake regulator
GAS	Group A *Streptococcus*
GCB	Granulomatous colitis boxer dog model
HARV	High-aspect ratio vessel
HPr	Histidine-containing PTS phosphotransferase
HtrA	High-temperature requirement A class protein
IBD	Inflammatory Bowel Disease

IgR	Immunoglobulin receptor
IL	Interleukin
Km	Kanamycin
LB	Luria Bertani media
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LSMMG	Low-shear modeled microgravity
MAPKK	Mitogen-activated protein kinase kinase
M-cell	Microfold cell
MOPS	Neidhardt's MOPS-based defined media supplemented with glucose
MscL	Mechanosensitive channel of large conductance
OD	Optical density
PAF	Platelet-activating factor
PBS	Phosphate-buffered saline

PBST	Phosphate-buffered saline supplemented with Tween 20
PCR	Polymerase chain reaction
PEP	Phosphophenol pyruvate
PFGRC	Pathogen Functional Genome Resource Center
PMN	Polymorphonuclear neutrophil
PsaA	Pneumococcal surface adhesin A
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
PTS	Phosphotransferase system
PVDF	Polyvinylidene fluoride
RT-PCR	Reverse transcription-PCR
SAM	Significance Analysis of Microarrays
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOPC	*S*-*o*-nitrophenyl-L-cysteine
Tc	Tetracycline

THY	Todd Hewitt media supplemented with yeast extract
TIGR	The Institute for Genomic Research
TNF-α	Tumor necrosis factor-α
Tn*phoA*	Transposon containing alkaline phosphatase gene *phoA*
TSA	Trypticase Soy agar
UC	Ulcerative Colitis
UspA	Universal stress protein A
XP	5-bromo-4-chloro-3-indolylphosphate

INTRODUCTION

Chapter 1: Low-Shear Modeled Microgravity and Bacteria

Microorganisms inhabit a vast range of diverse ecological niches. In order for bacteria to successfully survive and persist in such dynamic environments, they must be able to adapt to constantly changing conditions such as temperature, pH, oxygen and nutrient availability, and to changes in physical forms of environmental stresses such as fluid shear forces.

MICROBIAL RESPONSES TO MECHANICAL STIMULI

"Mechanotransduction" refers to the process by which cells sense and respond to mechanical signals. There is increasing evidence supporting mechanical forces as important signaling regulators in cellular processes involving changes in biochemistry, gene expression, and tissue development. Eukaryotic cell molecules associated with these responses include cytoskeletal structures, transmembrane integrin receptors, and adhesins which interact with the extracellular matrix (70). Integrins and surface adhesion molecules (e.g. cadherins and selectins) function as receptors which sense changes in mechanical stresses outside the cell surface. These "mechanoreceptors" then transmit these signals from the membrane into the cell, inducing cytoskeletal rearrangements, which alter cell structure and mechanics. In contrast, information is limited regarding the responses of prokaryotic cells to mechanically-based stresses. Previous studies have shown that bacteria do contain specialized structures such as transmembrane channels which allow them to respond to environmental changes impacting cellular mechanics, such as shifts in osmotic conditions. Sukharev *et al.* characterized the molecular mechanism of action for the mechanosensitive channel of large conductance (MscL) found in *Mycobacterium tuberculosis* and *Escherichia coli* (133). The MscL channel functions as a cytoplasmic gate which opens to relieve turgor pressure in response to

stress tension applied to the membrane due to hypo-osmotic conditions encountered in the environment. This mechanosensitive channel represents the first example of an isolated bacterial molecule shown to respond to mechanical stimuli by going through a conformational change, resulting in the opening of a large aqueous pore (132).

Other prokaryotic mechanosensitive structures include the collagen receptor of *Staphylococcus aureus* and the FimH adhesin of *E. coli*, both of which have previously been shown to alter the adherence properties of these two bacterial genera in response to changes in fluid shear forces. Using a parallel plate-flow chamber system, Mascari *et al.* showed that the detachment rate by collagen adhesin (CNA) of *S. aureus* decreases in response to increased fluid wall-shear rates (90). Flow chamber systems are essentially composed of two microscope slides stacked on top of one another and separated by a rubber or silicone gasket creating a space between the slides to act as the flow chamber. Inlet and outlet ports are secured into opposite ends of the chamber, where holes are pre-drilled into the upper slide and function in the perfusion of liquids and/or suspensions of cells via a mechanical syringe drive. Flow rates are adjustable by altering the speed of the plunger or by the use of syringes with different capacity limitations. Adherence / detachment rates are visually recorded with a video camera-mounted microscope and mathematically calculated based on counts of adherent cells at different times (53). The authors suggested that the decreased adherence, in response to increased fluid shear forces, could be attributed to unique intrinsic properties of the specific CNA bond, resulting in a strengthened bond. In a similar study, Thomas *et al.* elucidated the molecular mechanism of action by the FimH subunit (adhesin) of *E. coli* in response to increased fluid shear forces generated in a comparable flow chamber model (140). The FimH adhesin is one of three terminal subunits (FimF and FimG) located at the end of type I fimbrae associated with mannose-based adhesion. FimH is comprised of two domains: the lectin domain involved in the binding of mannose and the pilin domain which anchors the adhesin to the fimbrial tip. A ten-fold increase in fluid shear forces was shown to enhance bacterial adhesion to guinea pig erythrocytes by altering the conformation of FimH. The conformational change involved the extension of an interdomain linker chain connecting the lectin and pilin domains of the adhesin.

Explanations by the authors for the increased adherence included increased FimH-receptor bond formation and/or the initiation of a high-affinity conformation of the receptor-bound adhesin in response to elevated fluid shearing. Nickerson *et al.* previously proposed a theoretical model in which a potential surface-expressed "mechanosensitive" receptor undergoes a similar conformational change as that observed by the FimH adhesin in response to changes in fluid shear forces encountered by bacteria in the environment allowing the conversion of the external mechanical forces into an intracellular signaling phenomenon (Fig.1) (101).

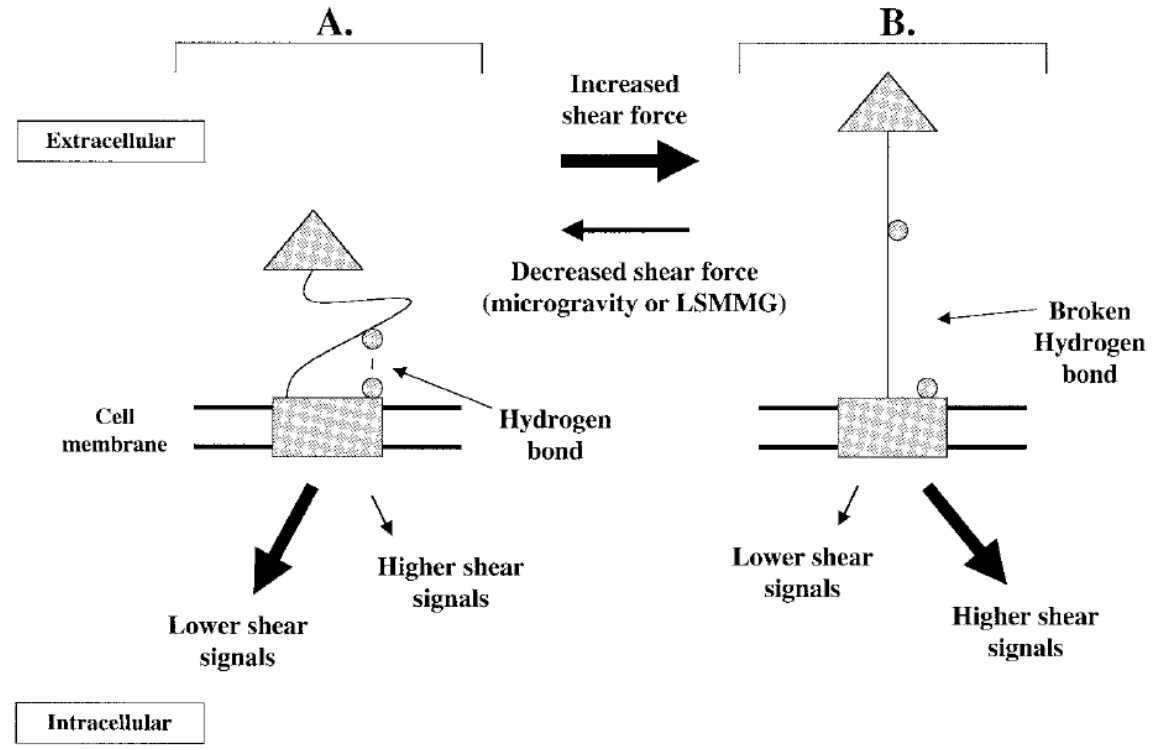


Fig. 1. Theoretical model illustrating a potential mechanism used by microbes to sense changes in aqueous shear forces. (A.) low-shear modeled microgravity and (B.) high fluid shear convert the external changes into a signal at the molecular level (reproduced with permission by Nickerson *et al.* and The American Society for Microbiology).

MODELING LOW-SHEAR MODELED MICROGRAVITY (LSMMG) CONDITIONS WITH HIGH ASPECT RATIO VESSELS (HARVS)

Changes in fluid shear forces are commonly encountered by pathogenic bacteria during the colonization of different niches within the human host. These fluctuations can range from low shear forces (< 1 dyne/cm^2), like those encountered *in utero* and between brush border microvilli of epithelial cells to higher forces (4-50 dynes/cm^2), as encountered along the walls of blood vessels (31, 64, 130). A number of different rotating-wall bioreactors have been utilized as model systems to generate environments characterized by low shear and low turbulence to cultivate cell cultures in a suspended state. Within this family of bioreactors are the high-aspect ratio vessels (HARVs) which were originally designed by the NASA Biotechnology Group based at the Johnson Space Center, Houston, TX, for the purpose of studying cell cultures under microgravity conditions (66).

HARVs are basically cylindrical vessels which contain three ports (1 large fill port and 2 small sampling ports) on the front side of the vessel and a hydrophobic membrane on the back side to allow for gas exchange. HARVs are completely filled with fluid (e.g. media) resulting with zero headspace to minimize fluid shearing due to the absence of air bubbles. Cultures are maintained in a suspended state due to the continual rotation of the vessels at an optimized speed (preventing sedimentation and centrifugation) with the vessel positioned such that the axis of rotation is perpendicular to the gravitational vector (Fig. 2A). The appropriate control is typically a second rotating HARV positioned with the axis of rotation parallel to the gravity vector such that the cell culture now experiences normal gravitational shear forces (Fig. 2B). The low fluid shear levels generated in HARVs have previously been measured quantitatively through mathematical modeling and were shown to generate levels < 1 dyne/cm^2 which correlate with physiologically relevant levels experienced *in vivo* (64, 97).

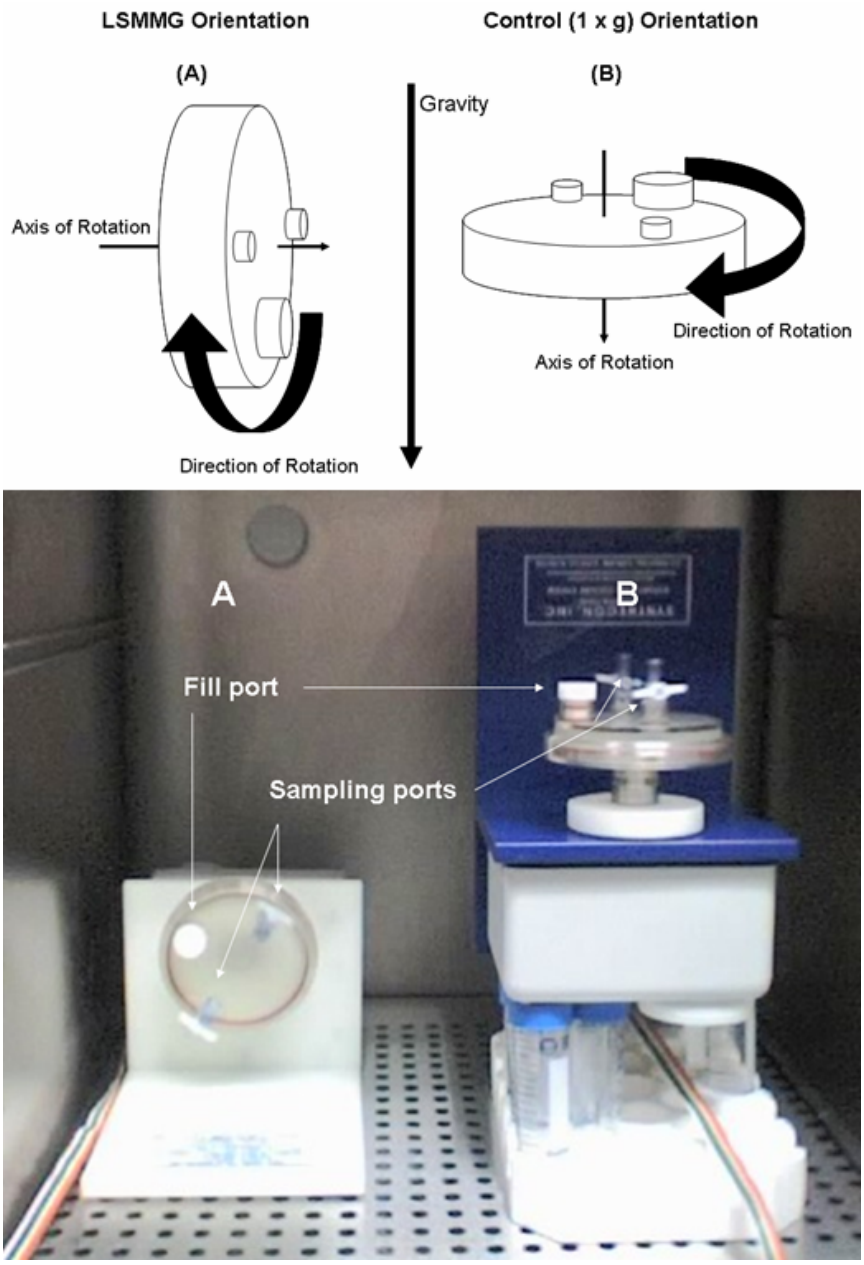


Fig. 2. Operational positions of High-Aspect Ratio Vessels (HARVs) in LSMMG and control (1 x g) orientations. In the LSMMG orientation the axis of rotation is perpendicular to the gravitational vector (A) while in the control (1 x g) orientation the axis of rotation is parallel to the gravitational vector (B) allowing growing cultures to experience normal gravitational shearing conditions.

MICROBIAL RESPONSES TO THE LSMMG ENVIRONMENT

Previous studies utilizing HARVs to cultivate different bacterial genera under LSMMG conditions have reported changes in many aspects of microbial biology, such as alterations in physiology, gene and protein expression, and pathogenesis. Some of the earliest studies examining the effects of LSMMG on bacteria showed that cultures grown in the HARVs undergo alterations in certain aspects of secondary metabolism such as antibiotic production and localization. The production of antimicrobial peptides such as cephalosporin, microcin B17, and rapamycin produced by *Streptomyces clavuligerus*, *E. coli*, and *S. hygroscopicus*, respectively, were found to be inhibited by cultures grown under LSMMG conditions, whereas the production of gramicidin S by *Bacillus brevis* was found to be unaltered (44-47, 56). Other observed metabolic changes under LSMMG conditions include the absence of glycerol-based repression of gramicidin S and microcin B17 production by *B. brevis* and *E. coli* normally observed in shaker flasks (45, 46). Changes in antibiotic localization have also been observed as a specific response to LSMMG conditions. Microcin B17 and rapamycin were found to accumulate in the media of HARV-grown cultures in contrast to the intracellular production of these antimicrobials by shaker flask cultures (44). The authors attributed the extracellular localization of these products to be dependent on fluid shear levels experienced by the bacteria, since introduction of a single glass bead into the HARV reversed the effects. The addition of synthetic beads of different sizes has been shown to quantitatively increase fluid shear forces generated within the HARVs (97).

In addition to alterations in the production of microbial products, LSMMG conditions have also been shown to alter growth kinetics and the ability of bacteria to resist different environmental stresses. *Salmonella enterica* serovar Typhimurium growth kinetics revealed a decrease in generation time when grown in minimal media under LSMMG conditions, in comparison to growth in rich media such as Luria-Bertani broth (LB) (147). An increase in dry-cell weight was observed by *E. coli* grown in minimal media in the LSMMG environment (44). The increased growth kinetics under LSMMG

indicates that the proliferation of certain microbes is enhanced. This could contribute to more efficient colonization and subsequent infection in the host. Wilson *et al.* previously showed that *S.* Typhimurium demonstrated an enhanced ability to resist thermal, osmotic, and acid stress conditions after growth under LSMMG, while resistance to oxidative stress was found to be diminished (147). A diminished ability to resist thermal and acid stresses was also observed in *S. pneumoniae* strain TIGR4 after growth under LSMMG (2). In contrast, *E. coli* strain AMS6 was found to exhibit an increased resistance to both osmotic and acid stress conditions after growth under LSMMG (85). Interestingly, in both the *S.* Typhimurium and *E. coli* studies, mutant strains in the *rpoS* gene were generated to investigate the impact of this global stress regulator under LSMMG conditions. While environmental stress resistance was not altered between wild-type and *rpoS* mutant strains of *S.* Typhimurium, the *E. coli* mutant strain demonstrated a decreased resistance upon entry into stationary phase in the HARVs. The observed difference in stress resistance between the wild-type and *E. coli* mutant strains could be correlated to RpoS activity levels in relation to growth phase. Optimal RpoS activity is generally experienced during the latter stages of growth such as the transition from late-log to stationary phase. These findings indicate the impact and importance of low-shear environments on different physiological components within bacteria, and suggest that while growth under LSMMG conditions can induce metabolic changes in microbes; these changes are not universal and vary between different bacteria.

Changes at both the genomic and proteomic levels have also been reported in which both global transcriptional and translational activities of bacteria have been altered after growth in the LSMMG environment. Following microarray analysis, *S.* Typhimurium strains 14028 (22 responsive genes) and χ3339 (163 responsive genes) were shown to exhibit altered global gene profiles in a strain-specific manner after growth under LSMMG (23, 148). LSMMG-responsive genes were found to be differentially expressed (up- or down-regulated) in response to LSMMG compared to those in 1 x g control cultures. In both studies, the majority of LSMMG-responsive genes were down-regulated. Characterization of responsive genes indicated that they represented various functional categories physically located throughout the genome. A

number of the genes identified in the χ3339 strain were found in the same transcriptional operon or in physically-linked clusters. The expression of lipopolysaccharide (LPS) biosynthetic genes was found to be down-regulated and corresponded to decreased LPS protein expression under LSMMG (148). Wilson *et al.* also reported the presence of binding sites for the iron uptake regulator, Fur, in the upstream regions of several LSMMG-responsive genes. Further characterization of wild-type and *fur* mutant strains revealed the participation of Fur in acid stress resistance under LSMMG conditions. Interestingly, this study revealed that known virulence genes were either non-responsive or down-regulated in response to the LSMMG conditions. In contrast, Chopra *et al.* reported genomic alterations in *S*. Typhimurium strain 14028 under LSMMG conditions and found the up-regulation of certain stress-related and virulence-associated genes (23). The stress-response gene *dnaK*, which encodes the heat-shock protein Hsp70, was up-regulated under LSMMG and previously found to function in the stabilization of RpoS translation in response to environmental stresses (121). The *Salmonella* virulence gene *virK* was also found to be up-regulated. This gene has been found to function in membrane remodeling in the host and in actin-based motility (35, 96).

Additional studies investigating the impact of LSMMG on the transcriptional profiles of bacteria have also included investigations with *E. coli* K-12 strain MG1655 (1, 142). In a recent study, Tucker *et al*. found that growth of *E. coli* MG1655 in either rich (LB) or chemically defined (Neidhardt's MOPS-based defined media supplemented with 0.2% glucose; MOPS) media under LSMMG conditions revealed no specific gene groups which were strictly responsive to LSMMG conditions regardless of media type. LSMMG-cultures exhibiting altered gene expression in LB were found to be associated with cell envelope processes, while altered genes in MOPS participated in translational processes. In both studies, overall changes in transcriptional activity were reflected by the down-regulation of the majority of LSMMG-responsive genes.

Changes in protein profiles have been reported in *S.* Typhimurium (14028 and χ3339) and enteropathogenic *E. coli* (EPEC) strain E2348/69 in response to growth in the LSMMG environment (23, 99). In both studies the expression of a number of functional proteins were found to be altered (repressed or enhanced), in addition to the induction of

proteins expressed only in the LSMMG environment. Alterations in the two-dimensional protein profile of *S. pneumoniae* TIGR4 have also been observed in response to growth under LSMMG (unpublished data). These findings demonstrate that the responses bacteria undergo in the LSMMG environment at the transcriptional and translational levels not only differ between different genera, but can also differ in a strain-specific manner involving regulatory strategies and different molecular pathways.

Changes in virulence potential and pathogenesis have also been observed in bacterial cultures grown under LSMMG conditions. Infection of BALB/c mice with LSMMG-grown *S.* Typhimurium χ3339, via oral administration, resulted in a decreased time-of-death, decreased LD_{50}, and an increase in colonization efficiency in different tissues (99). In addition, these cultures also exhibited an increased ability to resist acid stress and survive within macrophages. The ability to invade and stimulate the production of tumor necrosis factor α (TNF-α; in macrophages) and stress-associated mitogen-activated protein kinase kinase 4 (MAPKK4; in epithelial cells) was enhanced by LSMMG-grown *S.* Typhimurium 14028 (23). Responses to growth in the LSMMG environment also resulted in virulence-based alterations of pathogenic strains of *E. coli*, including increased production of heat-labile toxin by enterotoxigenic *E. coli* (ETEC) strain 180, increased TNF-α production in macrophages infected by EPEC strain E2348-69, and enhanced expression of the adhesin intimin, in enterohemorrhagic *E. coli* (EHEC) strain 86-24 (20, 23). LSMMG-grown *S. pneumoniae* TIGR4 have been shown to exhibit increased capabilities to adhere to and invade respiratory epithelia as well as a decrease in LD_{50} in interperitoneally infected mice (unpublished data). While these various findings support the enhancement in virulence potential among different bacterial strains, there is currently little evidence explaining what changes are occurring at the molecular and physiological levels in bacteria to induce such phenotypic changes under LSMMG conditions. Furthermore, the majority of bacterial virulence genes found to be altered under these conditions have been found to be down-regulated.

Chapter 2: *Streptococcus pneumoniae*

CLINICAL FEATURES AND EPIDEMIOLOGY

Streptococcus pneumoniae is a Gram-positive, non-motile, facultative anaerobic, encapsulated diplococcus, which replicates forming variable chain lengths in liquid and on solid medium. Laboratory diagnostic features include the presentation of α-hemolysis on blood agar, the absence of catalase activity, solubility in bile salts, and optochin susceptibility (11, 69). *S. pneumoniae* colonies can undergo phase variation taking on two different forms of opacity. Opaque colonies are characterized by large, white, uniform domed-shaped colonies in comparison to the less-defined transparent colony phenotype. Transparent variants express higher levels of surface phosphorylcholine (component of teichoic acid; cell wall constituent) which promote increased adherence to host cells expressing cellular platelet-activating factor (PAF) receptor leading to enhanced colonization capabilities in areas such as the nasopharynx (59, 95). In contrast, opaque phenotypes exhibit increased capsule production which allows for increased survival in the bloodstream. Most *S. pneumoniae* clinical isolates express an anti-phagocytic capsule, though non-encapsulated isolates have been isolated (conjunctivitis cases). The capsule consists of repeating oligosaccharides covalently bound to the cell wall constituents' peptidoglycan and C polysaccharide (CPS). Differences in capsule polysaccharide composition contribute to the identification of 90 different *S. pneumoniae* serotypes. In addition to phase variation and capsular differences, *S. pneumoniae* also exhibits the natural ability to internalize DNA from other bacteria (competence), allowing the acquisition of new phenotypic traits (transformation), such as new virulence factors, antibiotic resistance, and the production of new serologically different capsules (capsule switching).

S. pneumoniae is a member of the normal flora which colonizes upper respiratory niches such as the nasopharynx and can be isolated from both healthy children (20-40%) and adults (5-10%) (95). In immunocompromised individuals, however, *S. pneumoniae* is opportunistic and can cause mild-to-severe infections and diseases. The global rate of

invasive pneumococcal disease is 15 in 100,000 individuals annually, with higher incidences of infection found among newborns, infants up to 2 years of age, and the elderly (49). *S. pneumoniae* is a major cause of bacterial meningitis and otitis media (common ear infection) as well as the leading cause of community-acquired pneumonia (95). Previous infections (e.g. respiratory viral infections) contribute to the establishment of pneumococcal disease onset by disrupting clearance mechanisms which normally eliminate bacterial colonization (e.g. congestion in the opening of the eustachian tube and/or fluid accumulation in paranasal sinus cavities).

VIRULENCE FACTORS AND PATHOGENESIS

S. pneumoniae contains a number of virulence factors, including a capsule, adhesins, antigenic cell wall constituents, and toxins, which contribute to disease. The anti-phagocytic capsule plays a crucial role as a virulence factor in immunoevasion. Besides the obvious role of allowing the bacteria to avoid opsonophagocytosis, the numerous variations in the chemical structure of the CPS capsule allow for up to 90 different pneumococcal serotypes helping avoid antibody-mediated immunity. As previously discussed, differences in the content and amount of capsule expressed on the bacterial surface can promote survival in different niches within the host. Lower capsular levels allow for greater adherence and invasion potential, as observed by transparent variants in the nasopharynx where opaque variants expressing higher amounts of capsule can avoid opsonophagocytosis more effectively within the bloodstream (59, 72, 95). One of the main pathological characteristics of pneumococcal disease is the initiation of aggressive inflammatory responses. Cell wall components such as peptidoglycan and teichoic acid contribute to the antigenic stimulation of these responses when released upon bacterial lysis by activating the complement pathway and stimulating the secretion of macrophage activators Interleukin-1 (IL-1) and TNF-α (57). In addition, complement activation leads to the production of pathway components (i.e. C3a, C4a, and C5a) which function as chemotactic factors. Complement clearance mechanisms, such as phagocytosis and action by the membrane attack complex, are neutralized by the

presence of CPS-disrupting interactions between surface-bound C3b and phagocytic cells due to the presence of the thickened cell wall (25, 117). Phosphorylcholine is expressed on the bacterial surface in two states: a free form and a form bound by choline-binding proteins. Of the bound forms of phosphorylcholine, choline-binding protein A (CbpA) was the first surface adhesin described and the most abundantly expressed. CbpA plays an important role in bacterial adhesion to human cells and has a key role in penetrating the blood-brain barrier. During initial nasopharyngeal colonization, *S. pneumoniae* binds by unknown bacterial lectins to epithelial cell surface carbohydrates (*N*-acetyl-D-glucosamine-β1-3 and β1–4 galactose) and sialic acid. CbpA binds polymeric immunoglobulin receptors (IgR) on mucosal epithelial cells and PAF receptors of activated endothelial cells to facilitate transport of bacteria into the blood stream and across the blood-brain barrier, respectively (111, 112, 115). Additional Cbps, such as Cbp D, E, and G, also play roles in adhesion (62).

Other choline-binding proteins involved in bacterial carriage and virulence include pneumococcal surface adhesin A (PsaA), pneumococcal surface protein C (PspC), pneumococcal surface protein A (PspA), and autolysin (59, 72, 95). PsaA shares homology with lipoprotein adhesins in other streptococci and functions as a permease in the manganese transport system. PsaA also functions in the regulation of pneumococcal adherence and virulence. PspC interacts with complement through the binding of factor H, leading to C3b degradation and preventing complement activation. PspC can also bind and neutralize secretory IgA antibodies. PspA is an important virulence determinant that functions to prevent C3b activation and subsequent complement activation by interacting with factor B. PspA also functions as a lactoferrin receptor for iron acquisition. Autolysin is involved in growth phase-dependent autolysis which is triggered as pneumococcal growth transitions from late-exponential to stationary phase. Lysis of the bacteria contributes to the inflammatory responses through the release of cell wall components and intracellular toxins such as pneumolysin. In addition to reports of growth phase-dependent induction, there have been others indicating autolytic induction through the action of pneumolysin, produced by the pneumococcus, and by human

lysozyme produced as a defense factor in response to infection and the onset of inflammation (9, 27).

S. pneumoniae produces several enzymes which aid in colonization and invasion. Hyaluronidase functions to degrade connective tissue to enhance bacterial dissemination into the blood stream through the disruption of the blood-brain barrier. *S. pneumoniae* also produces two neuraminidases, NanA and B. These enzymes function to enhance adherence and colonization by cleaving *N*-acetyl-neuraminic acid from mucin and sialic acid residues from glycoconjugates of host tissues, to decrease mucus viscosity and expose additional receptors for pneumococcal adhesion. Secretory IgA1 protease degrades host IgA1, which protects bacteria from type-specific antibody. The protease cleaves antibodies at the hinge region generating one Fc fragment and two Fab fragments. This activity allows the bacteria to avoid mucociliary clearance by coating the bacteria with Fab fragments, which prevents the binding of intact antibodies containing the Fc segment which binds mucus for removal (74). The production and subsequent release of the cytotoxin, pneumolysin, during cell lysis functions as a thiol-activated cytolysin which binds target cell membranes, oligomerizes, and forms pores in the membrane of host-cells resulting in cell lysis. Additional effects of pneumolysin include the disruption of ciliary motion on epithelia, prevention of mucociliary clearance, the inhibition of lymphocyte proliferation and antibody synthesis, as well as reduced neutrophil migration and bactericidal activity (51, 52, 104).

S. pneumoniae infections can remain localized or develop into more severe forms of disease such as meningitis. Infections are initiated from upper respiratory colonization sites (e.g. nasopharynx) and can disseminate into the middle ear and sinuses and access the bloodstream leading to bacteremia, and the subsequent breaching of the blood-brain barrier resulting in the infection of the choroid plexus. Upon dissemination into the subarachnoid space, an inflammatory response is initiated which is key to the disease etiology of meningitis (72, 95). Studies using rabbit infection models suggest that bacterial cell wall components, such as peptidoglycan and teichoic acid, elicit cerebral spinal fluid abnormalities associated with meningitis through inflammatory mediators such as IL-1, IL-6, TNF-α, and C5a (109). Symptoms associated with pneumococcal

pneumonia include fever, chills, shortness of breath, fatigue, sweats, and cough. Pneumonia occurs from the successful invasion of bacteria into the alveolar spaces where replication occurs, and the immune responses, characterized by complement activation and generation of vasoactive substances, result in accumulation of exudative fluid and white blood cells in the alveoli and septa of the lungs. Fluid filling the lungs can be radiographically viewed as consolidated areas. Complications, such as empyema (collection of pus within pleural space between lungs and pleura membrane), can arise from pneumococcal pneumonia, resulting in mortality exceeding 30% in some documented hospital cases despite aggressive therapy. Additional syndromes, which are rare, yet can still occur from pneumococcal infection, include peritonitis, endocarditis, osteomyelitis, epidural and brain abscesses as well as soft tissue infections (95).

STREPTOCOCCUS PNEUMONIAE GENOMIC CHARACTERIZATION

Bacterial pathogens can adapt to different anatomical niches within the human host through mediating differential gene expression in response to changing environmental stimuli. Microarray technology has become a useful tool in characterizing how these changes alter the global genomic profiles of diverse bacterial generas such as *B. subtilis*, *M. pneumoniae*, *S. gordonii*, and *Borrelia burgdorferi* in response to numerous environmental changes such as alterations in salinity, oxygen availability, temperature, pH, and *in vivo* conditions, respectively (110, 129, 144, 146, 149). Microarray analysis of different pneumococcal species such as *S. pneumoniae* and *S. pyogenes* have demonstrated changes in global transcriptional activity in response to different environmental variables including temperature, antibiotics, competence-induction, and species/strain variability (65, 103, 107, 113, 127). Studies of group A *Streptococcus* (GAS) and *S. pneumoniae* have revealed that the expression of a number of genes, which function in different physiological processes, can be altered in a temperature-dependent manner (103, 127). GAS cultures grown at 29°C demonstrated altered gene expression by 9% of genes, including those genes which encode transcriptional regulators, transporters, and iron homeostasis proteins. Though the

expression of few known GAS virulence genes was altered, 28 genes encoding proteins with different secretion signals were altered. The altered expression of these genes can subsequently affect the extracellular proteome which could include proteins used in host cell interactions thus contributing to the survival and virulence of the bacterium. In *S. pneumoniae*, Pandya *et al.* also reported changes in the expression of genes distributed throughout the genome in response to incubation at different temperatures (21, 29, 33, and 40°C). Hierarchical cluster analysis revealed gene groups exhibiting correlating changes in expression in a temperature-specific manner. The relevance of such findings demonstrates that during colonization of the host, bacteria, such as *Streptococci*, can respond to changing environmental stimuli, such as temperature, within different parts of the body (e.g. nasopharynx, 33°C; lungs, 37°C; 40°C blood stream during febrile response), through mediating differential gene expression. By altering the expression of certain genes, bacteria can subsequently alter the expression of different proteins which can aid in processes such as colonization, cell division, virulence, and immune system evasion to enhance their ability to survive and persist in the host.

Microarray analysis has been employed in the identification of genes responsible for antibiotic resistance by *S. pneumoniae*. Studies using wild-type and mutant *S. pneumoniae* strains with disruptions in the operon encoding the VncRS two-component system (aids in vanomycin resistance) have revealed VncS-dependent regulation of gene clusters involved in the import of "death peptide" signals resulting in autolysis in response to antibiotics (113). Besides antibiotic resistance others have reported differences in transcriptional patterns when using global analyses to study other *S. pneumoniae* processes (e.g. the induction of competence and when studying phenotypic differences) among various clinical isolates. Peterson *et al.* used microarray analysis to characterize the kinetics of *S. pneumoniae* competence gene expression during exponential growth *in vitro* (107). Results of the study revealed the induction times of different genes involved in the onset of natural competence, while 8 additional loci were also identified and found to be induced during the competence process. In an effort to study intra- and interspecies genomic variations, Hakenbeck *et al.* used microarray analysis to compare different isolates of *S. pneumoniae* with reference strains addressing

the acquisition of antibiotic resistance and variations in capsule serotypes (65). Up to 10% of the genes were altered between isolates and the reference strains and 10 clusters were identified which accounted for half of the altered genes. Most of the genes were associated with transposases and mosaic genes housing antibiotic resistance determinants. In addition, distinct antigenic profiles were observed upon overall comparison with commensal strains such as *S. mitis* and *S. oralis*. These reports indicate the importance of genomic profiling in understanding *S. pneumoniae* responses to different environmental stimuli as well as distinguishing the characteristics among different species and strains.

Chapter 3: Adherent-Invasive *Escherichia coli*

INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is characterized by the inflammation of the gastrointestinal tract and can be accompanied by a range of symptoms, including fever, abdominal pain and cramping, and repeating episodes of severe watery or bloody diarrhea. IBD is typically manifested by one of two different disease forms: ulcerative colitis (UC) or Crohn's disease (CD). UC is mainly localized to the inner lining of the rectum and the colon (large intestine). UC inflammation begins at the rectum and lower intestine (sigmoid area) and progresses upward, affecting continuous sections of the inner lining of the colon. These inflamed tissues can develop bleeding ulcers secreting mucus and pus. While there is currently no cure for UC, drug therapies can be implemented to reduce symptoms, and in severe cases surgical resectioning of the colon can be carried out to remove diseased sections of tissue (92).

Unlike UC which rarely involves the small intestine, CD inflammation typically begins at the lower region of the small intestine (ileum), but can also extend into additional areas including the rectum, stomach, esophagus, and mouth. CD is initially manifested as a scattering of small sores along the ileal lining which can become inflamed and develop into ulcers. Ulceration in the mouth and throat can complicate eating and swallowing, causing pain to patients. In contrast to UC, separate patches of inflamed sores can occur simultaneously in different regions leaving areas of healthy tissue in between. Large volumes of water and salt can be secreted by affected areas overwhelming the absorptive capacity of the colon, resulting in the development of frequent and repetitive diarrheal episodes. The continual inflammation and ulceration of bowel tissues can lead to the swelling and thickening of bowel walls and scar tissue disrupting the movement of fecal materials through the bowel and leading to abdominal cramping and pain. In addition, the growth of the sores into large ulcers can lead to penetration into, and sometimes through, digestive tract walls. As with UC, there is no

cure for CD, and options include drug therapies and surgical alternatives in severe cases. The onset incidence of IBD has been found to be highest among populations living in cities of industrialized countries, with equal representation among men and women. In the U.S. alone there are currently over 500,000 cases of UC, targeting individuals between the ages of 15 and 40 years of age. There are more than 100,000 cases of CD in children, typically diagnosed around the ages of 10 and 11, in the U.S. (92).

There is no known cause for either UC or CD; however contributing factors include hereditary factors, immune system dysfunction, environmental factors, and the use of antibiotics. Typically, 20% of CD patients have an immediate relative (parent, sibling, child) with some form of IBD, which suggests a hereditary link to the predisposition (92). High frequencies of CD patients have been shown to have mutations in the NOD2/CARD15 gene which is thought to be associated with the early onset of symptoms and high risk of post-surgical relapse (13). Aberrant and unregulated hypersensitivity by the immune system to either invading enteric pathogens or to normal flora within the gut is also thought to contribute to the chronic state of inflammation associated with IBD. Environmental factors thought to contribute to IBD pathology include the presence of bacterial, viral, and/or food antigens which can repeatedly stimulate immune responses leading to chronic inflammation. Lastly, the use of antibiotics is thought to contribute by disrupting the balance of microbial flora in the intestinal tract leading to the overgrowth of species which could contribute to colitis, such as that observed from overgrowth of *Clostridium difficile* (50). While all of these factors have the potential to contribute to the onset of IBD, increasing evidence suggests that abnormal immune responses to microflora in the gut contributes to the chronic inflammation of IBD and will be further discussed.

MICROBIAL FLORA AND CROHN'S DISEASE

CD has been characterized by the presence of chronic transmural, segmental, and granulomatous inflammation within the intestinal tract (43). The features presented in CD patients correlate with those of various microbial infections in the gut and can include

ulcerations, microabscesses, fissures, fistulas, granulomas, and lymphangitis (29). Pathogens such as *Mycobacterium sp.*, *Yesinia enterocolitica*, and *Chlamydia trachomatis* have been shown to induce inflammatory responses similar to that exhibited by CD patients (119). Previous studies have found evidence which suggests that the microbial flora within the gastrointestinal tract of CD patients could be functioning as an antigenic stimulus contributing to the chronic state of inflammation through unregulated immune responses by the host. Clinical studies conducted with CD patients have shown that a decrease in luminal floral concentrations in response to intestinal lavage and antibiotic treatment resulted with symptomatic improvements (116, 134). In rodent models of intestinal inflammation, symptoms were shown to be attenuated or absent when the animals were kept under germ-free conditions (122, 136). Cave *et al.* found that mice developed granulomas when inoculated with gastrointestinal homogenates prepared from CD patients (21). Various studies have shown correlations with the presence of bacterial pathogens prior to and during the manifestation of CD in patients. A small percentage of patients during epidemics involving *Shigella sp.*, *Yersinia sp*., and *Salmonella sp*. developed IBD without prior evidence of bacterial infection (108). In other cases, CD patients have either tested positive for various bacterial antigens (e.g. *Listeria*, *E. coli*, *Streptococcus sp.*) or had bacterial isolates (e.g. *M. tuberculosis*) successfully removed from tissues (22, 83).

E. COLI CONTRIBUTIONS TO CROHN'S DISEASE

E. coli is a motile, Gram-negative, aerobic bacillus which comprises a major part of the normal flora within the gastrointestinal tract. While the presence of non-pathogenic species of *E. coli* play an important role in the maintenance of normal intestinal physiology and luminal flora stability, there are a variety of pathotypes which cause a number of diarreheagic and extraintestinal diseases through the expression of different virulence factors affecting a range of cellular processes. While several different bacterial genera have been implicated in their contribution to CD etiology, increasing evidence suggests *E. coli* as a candidate in the contribution of disease. While altered

luminal flora concentrations have been found in CD patients, the neoterminal ileum has been found to be colonized by abnormally high levels of *E. coli* (81). Surgical sampling results have shown that the frequency of isolating *E. coli* from intestinal serosa and mesenteric lymph nodes was 27 and 33% higher in CD patients, respectively, in comparison to controls (4, 78). Analysis of the ileal mucosa of CD patients showed *E. coli* recovery frequency was 65% from chronic lesions and 100% from early lesions, while overall predominance was abnormally high (50-100%) (30).

In addition, clinical studies have shown *E. coli* antibody titers to be higher in CD patients compared to those of control groups; however no specific serogroups were associated with disease (17, 135). Using immunocytochemistry, Cartun *et al*. reported that 69% of CD patient mucosal samples were positive for *E. coli* antibodies, while Liu *et al*. reported that in 57% of CD cases, positively labeled tissues were located in the lamina propria, along fissures, and on macrophages localized beneath ulcers (19, 83). While no single serogroup or specific strain of *E. coli* has been identified from CD patients, Masseret *et al.* reported that CD isolates did share common ribotype patterns (91). These profiles have been found in both healthy and ulcerated mucosa suggesting uniform colonization regardless of the state of the tissue and the possibility of a common evolutionary ancestry.

CROHN'S DISEASE-ASSOCIATED *E. COLI* VIRULENCE PROPERTIES

Pathogenic *E. coli* classes contain well-characterized virulence factors which aid in the progression of infection and disease. These factors include a variety of adhesins, invasins, toxins, and secretion systems which aid in the attachment and infection of host cells. Clinical *E. coli* strains removed from stool samples and biopsies of CD patients have been investigated for the presence of virulence-factor encoding genes common to these pathogenic classes, and none of these genes have been identified in the isolates (30, 120). However, these clinical isolates have demonstrated intrinsic adherent and invasive properties, which play an important role in colonization and infection of host tissues and thus have been categorized in a new putative class of *E. coli* known as adherent-invasive

E. coli (AIEC) (29). Several studies have demonstrated the adhesive potential of AIEC to different epithelial cell lines. AIEC strains isolated from 53-62% of stool samples taken from CD patients have exhibited adherence to buccal cells compared to 5-6% of isolates from controls (18, 58). Comparative studies investigating the adherence properties of AIEC strains isolated from the ileal mucosa of CD patients reported that the strains preferentially adhere to differentiated Caco-2 cells, which are representative of mature intestinal epithelia (30). This preferential adherence trend correlates with findings in CD patients which show that crypt epithelia, which are representative of immature intestinal epithelial cells, are rarely involved in early lesion formation (118).

The primary lesions that develop in patients are indicative of early stages of CD (e.g. aphthous ulcers develop from necrotizing M-cells) and mainly occur in Peyer's patches (54). The formation of these lesions is similar to those developed during infections by enteric pathogens, such as *Shigella* and *Salmonella*, in which invasion of host cells plays an important role in their virulence. AIEC strains isolated from early and chronic ileal lesions of CD patients have been shown to exhibit efficient invasion capabilities. AIEC strain LF82 was previously characterized for its invasive potential and found to effectively invade a number of different epithelial cell lines *in vitro*, including Caco-2, HCT-8, HEp-2, and Intestine-407 cells (15). Comparative analysis of the invasive properties of AIEC LF82 revealed unique properties which differ from those of other invasive pathogens. Most invasive bacterial strategies investigated such as those of enteroinvasive *E. coli* (EIEC) and *S. flexneri* are dependent on actin microfilament functioning, but are independent of microtubules (26, 41). In contrast, AIEC LF82 uptake depends on both functional actin microfilaments and microtubules (15). The uptake mechanism resembles a macropinocytosis-like process which involves the elongation of host cell membrane extensions around the bacterial cell. Upon entry into the host cell, the bacteria lyse the endocytic vesicle and successfully survive and replicate within the host cell cytoplasm. The invasion process carried out by AIEC strains is unique in that none of the genetic invasive determinants possessed by other invasive pathogens have been found in these strains. Boudeau *et al.* previously investigated the invasion process carried out by AIEC LF82 and found that type 1 pili-mediated adherence played a key

role in the invasion of host cells (14). AIEC LF82 invasion was found to be inhibited by the presence of D-mannose or methyl-α-D-mannopyranoside. Adherence by type 1 pili to host cells is dependent on the binding of D-mannose moieties on the host cell surface. Non-invasive mutants generated by Tn*phoA* mutagenesis were found to contain disruptions in genes comprising the *fim* operon, encoding the type 1 pili, and were unable to induce membrane extensions upon adherence to host cells. The induction of membrane extensions and subsequent invasion by the mutant strains was restored upon trancomplementation with cloned *fim* operon of non-pathogenic strain *E. coli* K-12. Interestingly, complementation of non-invasive laboratory strain JM109 with the *fim* operon from LF82 did not transfer invasive capabilities, indicating that the type 1 pili must be expressed in the AIEC genetic background to confer invasive ability. In addition to type 1 pili, bacterial flagella were also found to function in the co-operation of AIEC LF82 adhesion and invasion directly through active motility and indirectly through the regulation of type 1 pili expression, respectively (7). More recently, absence of the expression of lipoproteins NlpI and YfgL were found to diminish AIEC LF82 adherence and invasion abilities. The contribution of NlpI expression was suggested to function in the regulatory pathways mediating the expression of flagella and type 1 pili, because an *nlpI* mutant AIEC LF82 strain was unable to express flagella and exhibited small amounts of type 1 pili (8). A *yfgL* mutant strain of AIEC LF82 was shown to exhibit a highly decreased ability to invade epithelia and release outer membrane vesicles which, previously, have been shown to function in the intercellular transport of virulence factors to the host cell (114).

Intracellular pathogens have been the main focus in identifying potential microbial sources contributing to CD due to their ability to evade phagocytosis-based killing, survive within macrophages, and function as antigenic stimuli for T cells and macrophages, in eliciting the chronic inflammatory state of the gut. One of the main histological hallmarks of CD is the development of granulomatous inflammation in the lymph nodes and epithelial lining of the intestinal tract. This etiology has also been found during infections by intracellular bacteria and fungi (29). Previous studies have detected *E. coli* antigens in lymph nodes, granulomas, and in macrophages of CD patients

(19, 83). Glasser *et al.* have previously reported AIEC strains to successfully invade, survive, and replicate within macrophages without inducing cell death (61). In these studies intracellular replication increased up to 74-fold when compared to initial infection and escape from phagosomal vesicles was not found to be a required step as seen in epithelial cells. Instead, the fusion of these early vesicles into larger vacuoles was observed during macrophage invasion as seen in *Salmonella* and *Y. enterocolitica* infections (3, 63). This activity has been thought to promote survival through the dilution and attenuation of toxic compounds within the phagosomes. In a recent study, Bringer *et al.* found that the high-temperature requirement A class (HtrA) protein played an important role in survival during intramacrophagic replication (16). Inactivation of the *htrA* gene in an AIEC mutant resulted in a decreased ability to resist oxidative stress and diminished growth capabilities under acidic and nutrient poor conditions, as experienced in the microenvironment of phagosomes. Thus, AIEC appears to contain specific genes, such as *htrA*, which play a key role in survival in response to unfavorable environments such as those encountered upon cell invasion, e.g., the acidic environment of macrophage phagosomes. One unique feature exhibited by AIEC strains is the ability to avoid the induction of macrophage cell death upon invasion, as opposed to other invasive bacteria. Classical indicators of cell death including DNA fragmentation/degradation, cell detachment, and lactate dehydrogenase (LDH) release have not been observed in AIEC-infected macrophages even up to 24 hours post-infection (61). The absence of macrophage cytotoxicity and death by AIEC uptake differ greatly from that observed by most of the diarrheagic *E. coli* pathovars which elicit cytotoxic events and apoptosis in infected macrophages (79, 80).

AIEC strains have been shown to be capable of the continual stimulation of infected macrophages to produce large amounts of pro-inflammatory cytokines (e.g. TNF-α) which can contribute to the chronic inflammatory state of the gastrointestinal tract of CD patients (61). The continual activation and production of TNF-α demonstrates that not only are the infected macrophages still active, but that this constant activation can be sustained through the replication of the intracellular bacteria residing in the fused vacuoles of the macrophages. The current working model for the contribution

of AIEC to CD pathogenesis involves the successful colonization and invasion of the intestinal mucosa followed by translocation across the intestinal barrier (29). Once across the barrier, bacteria are taken up by residential macrophages where they replicate within fused vacuoles and stimulate the continual production of TNF-α. The secretion of this cytokine stimulates intestinal epithelia to secrete large amounts of pro-inflammatory chemokines (e.g. IL-8) which, in turn, signal the recruitment of polymorphonuclear leukocytes (PMN) such as neutrophils (Fig. 3). Unregulated PMN recruitment can then lead to the subsequent disruption and increased permeability of the intestinal barrier, thereby promoting further bacterial invasion. The disruption of this important barrier would allow for further macrophage activation and bacterial penetration into deeper tissues which could induce granuloma formation and chronic intestinal inflammation both of which are representative of CD pathology. Previous studies have shown increased levels of IL-8 measured in the intestinal biopsies of CD patients (28, 94). Findings from different animal infection models have provided evidence supporting correlations between the presence of AIEC to persistent inflammation and infection. Simpson *et al.* reported that clinical isolates characterized from biopsies of granulomatous colitis boxer dog (GCB) models exhibited comparable phenotypic, phylogenic, and virulence profiles to that of AIEC prototype strain LF82 (126). *E. coli* strains associated with persistent intramammary mastitis were isolated and characterized from a bovine mastitis model and found to exhibit the intrinsic adhesive and invasive properties of AIEC strains (40).

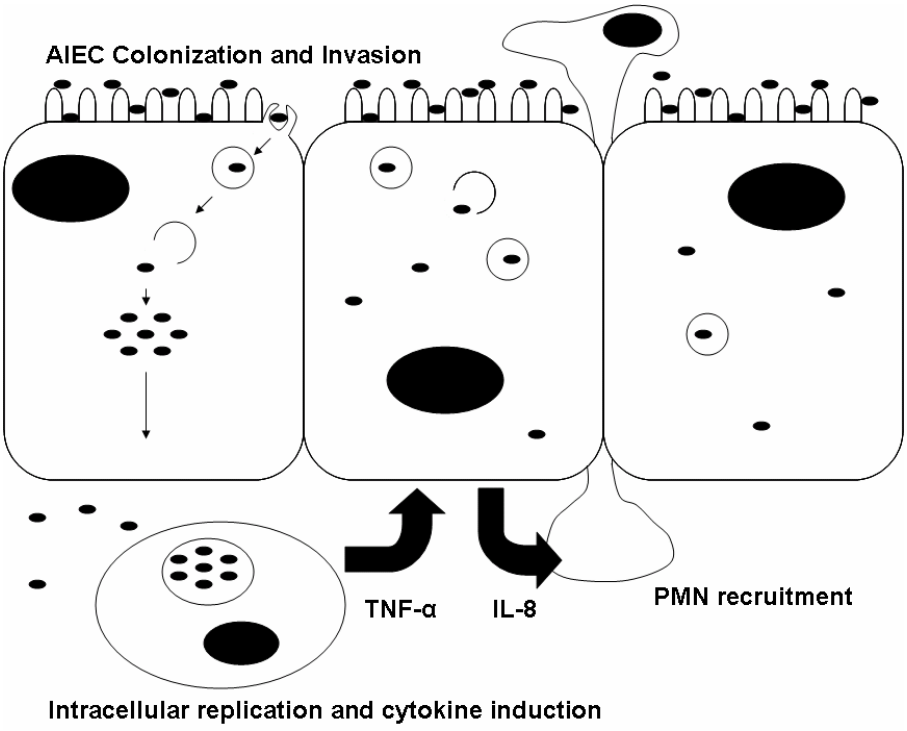


Fig. 3. Current model for AIEC participation in CD pathogenesis. Colonization precedes invasion into epithelia followed by uptake into residential macrophages. Intracellular replication stimulates the subsequent production of TNF-α which stimulates epithelia to secrete potent chemokine attractants such as IL-8 initiating polymorphonuclear (PMN) leukocyte recruitment.

Objectives of this Dissertation Study

The use of HARVs as a model system to study the effects of LSMMG on bacteria has produced important findings which show that bacteria are, indeed, responsive to mechanical stimuli in addition to other physiological forms of stress. These findings have been shown to impact various aspects of prokaryotic physiology, virulence potential, and activity at both the transcriptional and translational levels. The work presented here utilizes HARVs as a model system to characterize the effects of LSMMG on two very different bacterial genera, adherent-invasive *E. coli* (AIEC) and *S. pneumoniae*.

In Chapter 2, the use of microarray technology is discussed regarding its potential as a tool to better understand how *S. pneumoniae* adapts to various environmental factors which begins at the genomic level and can be observed through alterations of the genomic profile of the bacterium. As discussed in Chapter 1, microarray analyses of different bacteria generas to LSMMG conditions have thus far been limited to investigations of Gram-negative enteric strains such as *S.* Typhimurium and *E. coli*. My work investigates, for the first time, the effects of the LSMMG environment on the global transcriptional profile of an upper respiratory, Gram-positive pathogen. This genomic analysis and characterization will provide a better understanding of how mechanically-based stresses also affect the global transcriptional activity of respiratory pathogens. These findings will allow for further studies which can pursue the identification of key regulators and putative molecular mechanisms modulating the responses observed by *S. pneumoniae* to LSMMG conditions.

As discussed in chapters 1 and 3, the effects of LSMMG have been found to alter many different properties of both commensal and pathogenic strains of *E. coli*; however, there is presently no knowledge of how AIEC isolates respond to different forms of mechanical stress, such as low-fluid shearing, as encountered within the gastrointestinal tract of the host where colonization occurs. HARVs were utilized to characterize basic and fundamental properties of AIEC O83:H1 grown under LSMMG conditions such as

growth kinetics, stress resistance, and colonization potential as well as some of the molecular regulatory components associated with these processes.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Strains and plasmids are listed in Table 1. *E. coli* strains were routinely grown in LB broth or on LB agar at 37°C while *S. pneumoniae* TIGR4 (139) was grown in Todd Hewitt broth supplemented with 0.5 % yeast extract (THY) or on Trypticase Soy agar (TSA) supplemented with 5% sheep blood at 33^{o}C in a candle extinction jar. M9 minimal media (M9 minimal salts (Gibco BRL), 1 mM $MgSO_4$, 0.05% NaCl, 0.2% glucose, 0.1 mM $CaCl_2$; pH 7.0) was used to culture *E. coli* strains in the HARVs while THY was used for *S. pneumoniae* cultures at 37°C in 5% CO_2 (86). Isolated colonies from plates were used to seed overnight cultures (5 ml). Fresh media (100 ml) was inoculated (1:100) using overnight cultures and aseptically added to two sterile HARV vessels (50 ml / vessel) (Synthecon, Inc.). Additional media was added to each HARV to completely fill each vessel and eliminate the presence of any air bubbles. Vessels were oriented into either the LSMMG or 1 x g control orientations (Fig.2) and incubated at 37°C at rotational speeds of 15 rpm for *S. pneumoniae* or 25 rpm for *E. coli*. The rotational speed for *S. pneumoniae* was previously optimized in the laboratory of Dr. David Niesel to prevent gravitational sedimentation and centrifugation of bacteria. The rotational speed used for *E. coli* has been previously optimized by Nickerson *et al.* (85, 99) for *Salmonella* and Lynch *et al.* (96) for *E. coli*. For microarray studies a second 1 x g static control HARV was included to function as an additional filter to exclude genes which were found to be differentially expressed between 1 x g rotating and 1 x g static conditions. Allen *et al.* have previously shown that gene expression can be altered between static and rotating 1 x g controls independent of LSMMG conditions (1). Therefore, genes found to be differentially expressed between rotating and static controls were determined not to be directly impacted by the LSMMG environment and were excluded from further analysis. *S. pneumoniae* HARV cultures were monitored by optical density (OD_{600nm}) until cells entered mid-logarithmic phase (OD = 0.5) after

Table 1. Bacterial strains and plasmids

Strain / Plasmid	Relevant Characteristics	Reference
Strains:		
Streptococcus pneumoniae	Strain TIGR4 (serotype 4)	(139)
E.coli strains:		
AMS6	K-12, λ-, F-, Δ*lac*	(73)
AIEC O83:H1	clinical isolate from Crohn's Disease patient; Tc^r Ap^r Sm^r Cm^r	Dr. Alexander Swidsinski Charite Hospital, Germany
O83:H1 cu	Antibiotic sensitive isolate from AIEC O83:H1	This study
SM10 (λ *pir*)	*thi thr leuB tonA lacY supE recA::*RP4-2-Tc::Mu-Km; Km^r	(125)
CAA001	AIEC O83:H1cu *rpoS::cat*; Cm^r	This study
CAA003	CAA001::Tn*phoA* isolate 1; Cm^r Km^r	This study
LE392	F-, *hsd*R574 (rk-, mk+), *sup*E44, *sup*F58, *lac*Y1, *gal*K2, *gal*T22, *met*B1, *trp*R55	Promega
Plasmids:		
pBlueScript SK	Cloning vector; Ap^r	Stratagene
pCVD442	Suicide vector; Ap^r Sucrose sensitive	(42)
pRPO::Cm	*rpoS*::*cat* in pCVD442; Cm^r	This study
pRT733	*oriR6K mobRP4* Tn*phoA*; Km^r Ap^r	(137)
pGEM-T Easy	Cloning vector; Ap^r	Promega
pRpoS	*rpoS* in pGEM-T Easy; Ap^r	This study
pLAFR3	Broad-host-range cosmid vector; Tc^r	(128)
pMD1	*tnaC*, *tnaA*, and *tnaB* cloned into pACYC184; Tc^r	(34)

which samples were collected for experimentation. Harvesting *S. pneumoniae* cultures at mid-logarithmic phase avoids cultures from undergoing natural stationary phase-induced autolysis. *E. coli* HARV cultures were harvested after 12 h of growth and then quantitated based on spectrophotometric analysis. Concentrations were then adjusted for experimentation. When required, growth media was supplemented with antibiotics at the following concentrations: chloramphenicol (Cm) 30 μg ml^{-1}; kanamycin (Km) 50 μg ml^{-1}; ampicillin (Ap) 100 μg ml^{-1}; and tetracycline (Tc) 25 μg ml^{-1}.

Microarray analysis.

S. pneumoniae HARV cultures were grown to an optical density of 0.5, harvested and neutralized in a 2:1 volume (reagent to culture) of RNAprotect bacterial reagent (Qiagen, Valencia, CA) to stabilize RNA, cells were pelleted, and stored at -80°C. Prior to RNA isolation, bacterial cells were resuspended in lysis buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0, 1 mg/ml lysozyme) then lysed by mechanical disruption using a Mini BeadBeater™ (Biospec Products, Inc.) for 5 minutes at maximum speed with 0.1 mm zirconia/silica beads (Biospec Products, Inc.). RNA was isolated with RNeasy columns (Qiagen, Valencia, CA) and DNase treated using Turbo DNase-Free (Ambion, Austin, TX), according to manufacturers' instructions. Purified RNA was used to generate cDNA which was labeled and applied to *S. pneumoniae* strain TIGR4 genome cDNA microarrays, developed by The Institute for Genomic Research (TIGR, Rockville, MD), based on the TIGR4 strain genomic sequence, and provided by the Pathogen Functional Genome Resource Center (PFGRC) of the National Institutes of Health. Each microarray consisted of polymerase chain reaction (PCR) products representing segments of 2131 open reading frames (95% of predicted coding regions) from strain TIGR4.

The generation and labeling of cDNA, microarray hybridization and processing, and intensity value generation was performed by the UTMB Molecular Genomics Core facility (UTMB, Galveston, TX) as previously described (103). To generate reproducible and statistically significant data, duplicate microarray slides were used for each individual experiment. The cDNA generated from experimental and control samples was

labeled by the incorporation of either cyanine3-dUTP (Cy3; green fluorescence) or cyanine5-dUTP (Cy5; red fluorescence) fluorescently labeled nucleotides to distinguish competitive hybridization of array spots. In contrast to affymetrix array chips, each microarray slide was hybridized with differentially labeled cDNA from both control and LSMMG cultures. Three independent HARV experiments were performed comparing 1 x g control cultures with LSMMG while two independent experiments were carried out comparing different gravitational controls (1 x g rotating and 1 x g static conditions). The microarray data were analyzed using GenePix Pro 6.0 (Molecular Devices Corporation, Union City, CA), Spotfire DecisionSite 7.3 (Spotfire, Somerville, MA), Significance Analysis of Microarrays (SAM; Stanford University, Stanford, CA), and analysis of variance (ANOVA). Genepix Pro 6.0 is a standard microarray image analysis software suite which can be used to visualize the spots on microarrays, generate signal intensity values for each spot, and subtract background noise from arrays. Spotfire DecisionSite 7.3 is a software package containing a number of different analytic techniques and methods to assess and analyze microarray data as well as illustrate the data in different graphical forms (i.e. heat maps). SAM is a software program developed to identify statistically significant genes in microarray data (143). This is carried out by the assimilation of gene-specific *t* tests. Individual genes are assigned scores based on expression changes relative to standard deviations of repeated measurements. The expression of genes is deemed significant based on an adjustable threshold and the stringency of this threshold can be correlated with a false detection ratio (FDR) calculated as a percentage from permutations of the repeated measurements.

ANOVA is a multivariate statistic used to estimate the variance of a multiple-group population to determine statistical significance. Spotfire DecisionSite 7.3 was used to carry out ANOVA analyses. Gene expression changes of 1.5-fold or greater and with a p value of ≤ 0.05 were considered significant. Only genes whose expression was found significant by all four analysis techniques were considered to minimize the inclusion of false positives in the data. Computational hierarchical cluster analysis was carried out using CLUSFAVOR 6.0 (Baylor College of Medicine, Houston, TX), and Spotfire

DecisionSite 7.3 (Spotfire, Somerville, MA). A detailed description of these methods has been previously published (55).

Quantitative Real Time RT-PCR Analysis.

Results from microarray analyses were confirmed by performing quantitative real-time reverse transcription-PCR (RT-PCR) on seven representative genes found to be differentially expressed by LSMMG above threshold levels established for detection. Reactions were performed on the LightCycler thermal cycler system (Roche Diagnostics, Indianapolis, IN) using SYBR Green I dye (Qiagen, Valencia, CA) according to manufacturer's instructions. Reactions were prepared containing 200 ng of RNA, 1 μg of each primer, 10 μl SYBER Green PCR master mix, and 0.2 μl of reverse transcriptase in a total reaction volume of 20 μl. Standard thermocycler program settings included reverse transcription at 55^{o}C for 20 min and a denaturation step at 95^{o}C for 15 min followed by 40 cycles with 95^{o}C denaturation for 15 sec, 55^{o}C annealing for 20 sec, and 72^{o}C extension for 20 sec. The temperature transition rate was set at 20^{o}C per second. Detection of the fluorescent product was performed at the end of the extension period at 80^{o}C for 10 sec. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis. Negative controls containing water instead of RNA were concomitantly run to confirm that the samples were not cross-contaminated. Targets were normalized to reactions performed using the housekeeping DNA gyrase subunit B (*gyrB*) gene amplimers, and fold change was determined using the comparative threshold method (84). All of the experiments were performed at least in triplicate. Where applicable, the data were plotted as arithmetic mean ± standard deviation, and *Student's t*-test ($P \leq 0.05$) was used for data analysis.

Table 2. Quantitative Real-Time RT-PCR *S. pneumoniae* Genes and Primers

Locus	Gene	Primers
SP0553	*nusA*	(forward) 5'-AATTCCACCCAGCTCGTTACG-3'

		(reverse) 5'-AGCAGGAGCGATGGCATTGTA-3'
SP0575	putative helicase	(forward) 5'-CCAAAGTCCGAGAGAGAACGA-3' (reverse) 5'-TGCTGCCAAGATAAGCGTGTC-3'
SP1985	*ksgA*	(forward) 5'-AGAAAGAAGTAGCGGACCGCA-3' (reverse) 5'-AAAGACCGTACGAGGCACGAT-3'
SP1996	Universal stress protein, *uspA*	(forward) 5'-TCGCAATCGATGGTTCTAAGG-3' (reverse) 5'-GCGTGTGTCAATGACATGTGC-3'
SP0241	ABC transporter	(forward) 5'-TGCTTCAAGAAACCTGGCTCA-3' (reverse) 5'-TGGTATCGTATCCCTGTGGCA-3'
SP1096	putative adenylate cyclase	(forward) 5'-TTGACACTCAAAGTCCCGCAG-3' (reverse) 5'-TGTTTCGCCAATTCATCCAG-3'
SP0545	immunity protein, *blpY*	(forward) 5'-AGCATTCGCGATTTGGTCTT-3' (reverse) 5'-GCACCGCATAGGATGGATAAA-3'

Recombinant DNA Techniques.

Plasmid DNA was isolated from overnight cultures using the Qiagen QIAprep™ plasmid preparation kit (Qiagen, Valencia, CA) according to the manufacturer's procedure. Standard methods were used for restriction digestions, ligation and transformation of plasmid DNA, and chromosomal DNA isolation (86).

Tn*phoA* mutagenesis.

Insertional Tn*phoA* (transposon with alkaline phosphatase gene *phoA*) mutations were generated through conjugation of CAA001 and a SM10 (λ *pir*) strain containing the Tn*phoA* transposon on the suicide vector pRT733 (67, 125, 137, 138). Exconjugants from the mating were selected on LB plates supplemented with kanamycin, chloramphenicol, and the alkaline phosphatase substrate XP (5-bromo-4-chloro-3-indolylphosphate) (20 μg ml^{-1}) (Sigma Chemical, St. Louis, MO). Disruption of a gene

encoding a membrane-associated protein (i.e. secreted or cytoplasmic membrane proteins) would be indicated through the enzymatic activity of alkaline phosphatase which cleaves a phosphate group from XP (colorless substrate) resulting with a blue colored by-product and a subsequent blue colony. Disruption of the gene generates a gene fusion composed of the amino-terminal of the target gene fused to the *phoA* gene which encodes alkaline phosphatase which only exhibits high activity levels outside of the cytoplasm when exposed to the periplasmic space.

Cosmid library construction.

To locate the transposon insertion sites, a cosmid library was generated using the broad-range cosmid vector pLAFR3 (128). Genomic DNA was isolated by the modified Marmur protocol (88) and partially digested with *Sau3A1* according to Maniatis *et al.* (87). Digests which yielded fragments of 9-12 kb in size on 1% agarose gels were used in overnight ligation reactions (0.3 μg vector, 0.8 μg DNA, 60U ligase) containing *BamH1* digested pLAFR3 and T4 DNA ligase (New England Biolabs) at 16°C. Ligation reactions were introduced into *E. coli* strain LE392 by infecting the bacteria with phage particles packaged *in vitro* using the Packagene Lambda DNA Packaging System (Promega) according to the manufacturer's instructions. Bacterial colonies were selected on LB agar supplemented with Km and Tc. Cosmid DNA from isolates containing Tn*phoA* within the DNA cloned was sequenced by the UTMB Protein Chemistry Laboratory using a reverse primer flanking the 5' region of the transposon (5'-GTTTTCCAGAACAGGGCAAA-3'). Sequence results were analyzed using the Basic Local Alignment Search Tool program (BLAST) to compare against known nucleotide sequences in the EMBL data bank (http://www.ncbi.nlm.nih.gov/BLAST/) (105).

Construction of AIEC O83:H1 *rpoS* isogenic mutant by allelic exchange.

The *rpoS* gene (Entrez Accession No. NC000913) was PCR amplified in two segments: the 5' portion of the gene as a 922-bp fragment containing flanking *KpnI* (5' end) and *SmaI* (3' end) restriction sites and the 3' portion as a 904-bp fragment

containing flanking *SmaI* (5' end) and *BamHI* (3' end) restriction sites. The following two primer sets were used to generate the two fragments: 5RPO (5'-AACGGTACCAACAGCAAGC-3') and 3SMRPO (5'-GTCCCGGGTCAAACTTCTCTAC-3') for the 5' segment; and 5SMRPO (5'-GACCCGGGACGTGGTTTCCGCTTCTCA-3') and 3RPO (5'-GTCCCGGGTCAAACTTCTCTAC-3') for the 3' fragment. Each fragment was individually cloned into pBluescript SK, re-digested, and the two fragments ligated into the same plasmid. The cloned gene was disrupted with the *cat* gene inserted into *SmaI* restriction sites and the disrupted gene then subcloned into suicide vector pCVD442. The construct was introduced into AIEC O83:H1cu by conjugation with donor strain SM10 (λ *pir*) and allelic exchange was performed as previously described (141). Strain O83:H1cu is an antibiotic sensitive strain and displayed an identical adherent and invasive phenotype to the parental strain O83:H1 (data not shown). Candidate colonies which were lactose positive on MacConkey plates and resistant to chloramphenicol were screened for sensitivity to ampicillin and kanamycin. The presence of the *cat* cassette within the chromosomal *rpoS* gene of strain CAA001 was confirmed by PCR.

Transcomplementation of *rpoS* mutant.

The *rpoS* gene and its promoter region were amplified from the chromosomal DNA of AIEC O83:H1 using primers 5RPOS (5'-ACCAACAGCAAGCACAAC-3') and 3RPOS (5'-TGAGACTGGCCTTTCTGAC-3'). The PCR product was gel extracted, digested with *EcoRI* then ligated into *EcoRI*-linearized pGEM-T Easy. The resulting construct pRpoS was used to complement Tn*phoA* mutants generated from the RpoS mutant background. This complementation was used to determine if the disrupted genes in the identified Tn*phoA* mutants, containing disruptions in genes encoding membrane-associated proteins, were under the regulation of RpoS. Such mutants were identified by the loss of alkaline phosphatase activity upon complementation with an intact *rpoS* gene which resulted with the reversion of the blue colony phenotype (used to select Tn*phoA* mutants) back to a colorless colony phenotype.

SDS-PAGE and Western blot analysis.

Bacterial samples were removed from HARVs at 4, 12, and 24 hours, and cell concentrations were standardized based on OD_{600} readings. Cells (4×10^8) were pelleted, suspended in Laemmli protein sample buffer (77), boiled for 5 minutes, and used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cell lysates (10 µl/well) were loaded onto a 10% SDS polyacrylamide gel and run at 100 volts. Samples were transferred onto polyvinyl fluoride (PVDF) Immobilon-P transfer membrane (Millipore, Bedford, MA) and blocked in 5% non-fat milk overnight at 4°C. Membranes were washed with phosphate-buffered saline (PBST) with 0.1% Tween 20 and probed with a polyclonal anti-RpoS antibody kindly provided by A. Matin (Stanford University). Blots were developed with an ECL-Plus kit (Amersham, Piscataway, N.J.). Twenty-four hour AIEC O83:H1 and *rpoS* mutant test tube cultures were included as positive and negative controls.

Growth kinetics under LSMMG.

Cultures of *E. coli* AMS6, AIEC O83:H1, and the *rpoS* mutant were grown in M9 minimal media within HARVs oriented in LSMMG and 1 x g control positions as described in Figure 1. Growth of both strains was monitored by measuring $OD_{600\ nm}$ at one hour increments for 12 hours. Growth was monitored quantitatively by serial dilution and plating at 4, 8, and 12 hours.

Environmental stress assays.

HARV-grown cultures were harvested aseptically through the sampling ports of the LSMMG and 1 x g control vessels after 12 hours of growth and culture densities were determined by measuring optical density (OD_{600}). Bacterial cultures were grown in HARVs allowing an adequate amount of time to acclimate and take on phenotypic characteristics in response to LSMMG and 1 x g control environments within the vessels prior to stress challenges. A bacterial concentration of $1x10^7$ CFU (approximately 5-15

μl) was immediately exposed to one of the four different environmental stresses investigated. Specific stress levels, concentrations and exposure periods tested were comparable with those conditions previously tested for *S.* Typhimurium and *E. coli* AMS6 HARV studies (85, 147). To reduce variability in the results obtained with the HARV model system, *E. coli* K-12 strain AMS6, previously examined by Lynch *et al*., was included in all stress assays to function as a comparative bacterial control along with the strains examined (85). Thermal stress was evaluated by adding bacterial aliquots to 1 ml of LB liquid media then transferring cultures to heating blocks set at 55°C to heat shock the bacteria. Responses to acid, osmotic, and oxidative stresses were analyzed by adding bacteria to 1 ml of filter-sterilized media which was either pre-adjusted to a pH of 2.0 with HCl, supplemented with NaCl to a concentration of 3.0 M, or supplemented with H_2O_2 (Fisher Scientific) to a concentration of 10.0 mM. Stress exposure time was evaluated at 30 min and 1 hour, followed by serial plating in duplicate on LB agar plates to determine the percentage of survival. The use of LB as the stress diluent did not affect the growth kinetics of the cultures during the challenge periods since similar results for the assays were also observed using sterile phosphate buffered saline (PBS) as the diluent in subsequent studies. Cultures from each HARV vessel were serial diluted and plated at time zero prior to stress exposure to calculate percent survival. The percentage of survival was calculated by dividing the number of colony forming units (CFU) at either 30 min or 1 hour by the CFU at time zero and multiplying by 100. A minimum of three independent stress assays was carried out for each stress examined. Statistical significance ($p \leq 0.05$) was determined using *Student's t*-Test.

Bacterial adhesion and invasion assays.

T-75 flasks of Caco-2 cells were grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM) (Cellgro) supplemented with 10% (v/v) fetal bovine serum at 37°C under 5% CO_2. Cells were trypsinized, diluted, and seeded at $1x10^5$ cells ml^{-1} per well in 24-well plates (Corning) and incubated for 48 hours prior to infection. Before infection, monolayers were washed with sterile PBS (pH 7.4) and replenished with 1 ml

of DMEM without supplements. Monolayers were incubated with $1x10^7$ bacteria (MOI 100:1) per well for 1.5 hours at 37°C in 5% CO_2. Infected monolayers were washed twice with PBS to remove non-adherent bacteria followed by lysis with 200 µl of 0.1% Triton X-100 in PBS to recover adherent bacteria. Infected monolayers were incubated an additional hour in fresh media supplemented with gentamicin (50 µg/ml), to kill extracellular bacteria, prior to harvesting. Incubation times were optimized from standard 3 hour incubation periods to minimize time for culture re-adaptation outside of the HARVs while allowing sufficient time for interaction between bacteria and tissue culture cells. Supplementary assays carried out with incubation periods at 4°C confirmed adherence and invasion trends under LSMMG conditions. Incubation assays at 4°C excluded potential interference of metabolic and physiological changes occurring due to re-adaptation outside of LSMMG environment. Lysates were serial diluted and plated on LB agar to quantitate adherent bacterial numbers from triplicate wells as the mean**.** Statistical significance ($p \leq 0.05$) was determined using *Student's t*-Test.

Tryptophanase enzyme assays.

Tryptophanase activity was determined by measuring the conversion of *S-o*-nitrophenyl-L-cysteine (SOPC) to *o*-nitrothiophenylate (131). The assay procedure was based on those described previously (5, 60). Cells were grown to mid-logarithmic phase (OD_{600} = 0.5 - 0.6) in M9 minimal media, washed with and resuspended in 2 ml of cold lysis buffer (100 mM potassium phosphate, pH 7.8, 50 µm pyridoxal phosphate, 10 mM β-mercaptoethanol, and 2 mM ethylenediamine tetraacetic acid [EDTA]), and sonicated for 1 minute on ice. Lysates were spun down (12,000 x *g* for 2 minutes) to remove cells debris and protein concentrations from the supernatant determined using a Bio-Rad Protein assay reagent (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Fifty microliters of sample supernatant was combined with 450 µl of assay buffer (100 mM potassium phosphate (pH 7.8); 100 µm pyridoxal phosphate; 20 mM β-mercaptoethanol; and 1 mg bovine serum albumin per ml) and incubated at 30°C for 5 minutes. The reaction was started by the addition of 500 µl of pre-warmed 0.66 mM

SOPC. Reactions were monitored at 370 nm with a spectrophotometer over the course of 3 minutes until the ΔA_{370} calculated was linear. Enzymatic activity was calculated using an extinction coefficient of 1.86 M^{-1} cm^{-1} according to Suelter for *o*-nitrothiophenolate (131). Tryptophanase activity was calculated as follows: (ΔA_{370} x 1.0 ml reaction volume)/{(1.86 μmol/ml) x ([time (min)] x [protein concentration (mg/ml)] x [cell extract volume (ml)]} as described by Gish (60). One unit of tryptophanase-specific activity was defined as 1 μmol of *o*-nitrothiophenolate produced per minute at room temperature (~25°C). All extracts were assayed in duplicate.

RESULTS AND DISCUSSION

Chapter 4: The Effects of Low-Shear Modeled Microgravity on *Streptococcus pneumoniae* Global Gene Expression

INTRODUCTION

Bacteria grown under LSMMG conditions have been shown to exhibit alterations in physiology and increased virulence potential (100, 101). To better understand the genomic changes taking place influencing these changes in phenotype, microarray analysis has been utilized to examine the global transcriptional profiles of different enteric bacterial species including *S.* Typhimurium and *E. coli* (23, 142, 148). While these genomic profiles have aided in allowing us to better understanding how changes in gene expression under LSMMG conditions can affect Gram-negative enteric bacteria in terms of virulence and physiological adaptations, the effects of this unique environment have yet to be investigated in a Gram-positive species such as the upper respiratory pathogen, *S. pneumoniae*. Previous findings from the laboratory of Dr. David Niesel have shown that the growth of *S. pneumoniae* under LSMMG conditions can enhance virulence potential and alter both physiology and protein profiles (unpublished data). Therefore, microarray analysis was utilized to investigate the changes which take place at the genomic level in *S. pneumoniae* after growth under LSMMG to identify genes which are responsive to this environment and function in changes involving physiology, protein expression, and virulence potential previously observed.

RESULTS AND DISCUSSION

Cluster analysis reveals similar expression patterns in LSMMG-responsive genes in *S. pneumoniae*.

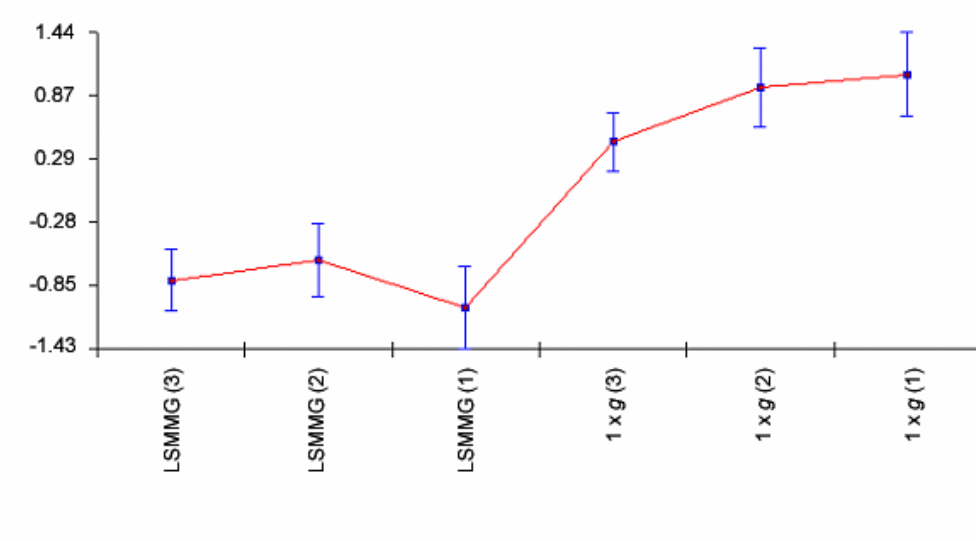
In an effort to identify trends in the global gene expression of *S. pneumoniae* in response to the LSMMG environment, hierarchical cluster analysis was carried out on

microarray results generated from HARV-grown cultures. A stringent analysis was incorporated to normalize and analyze microarray data using four independent analytical approaches. These included ratio calculations of normalized intensity values using GenePix Pro 6.0, pairwise comparisons using Spotfire DecisionSite 7.3, Significance Analysis of Microarrays (SAM), and analysis of variance (ANOVA). The threshold criteria for determination of significance included a minimum change in gene expression of 1.5-fold across all techniques and a p value of ≤ 0.05 for ANOVA statistics. Microarrays were scanned using GenePix Pro 6.0 to generate signal intensity values for array spots representing individual genes on the array. The values were adjusted to filter out background noise from arrays, and ratios were generated for each spot from the two signal intensity values generated representing the two hybridized samples. These ratios represented fold changes of genes differentially expressed in the 1 x g control versus LSMMG samples (Table 4). The intensity values generated by GenePix Pro 6.0 were used for hierarchical cluster analysis using CLUSFAVOR 6.0 and Spotfire DecisionSite 7.3 to identify trends in gene expression between the various samples (Fig. 4). Results from both clustering programs indicated an overall trend of down-regulation of genes grown under LSMMG conditions compared to that in 1 x g controls. Further analytical methods were carried out to identify statistically significant changes in LSMMG-responsive gene expression.

Identification of significantly altered genes under LSMMG by SAM analysis.

SAM analysis was carried out to identify genes whose expression changes in response to LSMMG were statistically significant. The intensity values between individual arrays were normalized by the mean, using Spotfire DecisionSite 7.3. These normalized values were then used in Spotfire DecisionSite 7.3 to carry out pairwise comparisons, and ANOVA analyses to filter out inconsistencies in gene expression differences among replicates and to generate fold-change and p-values for those genes exhibiting consistent and reproducible changes in expression (Table 4). The normalized intensity values were also used in SAM to carry out both paired and 2 class unpaired,

A.



B.

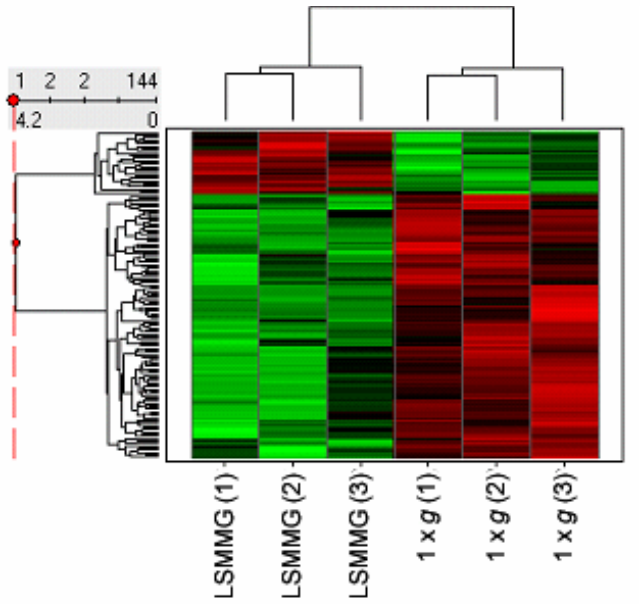


Fig. 4. Hierarchical clusters of genes from 1 x g and LSMMG-grown *S.* p*neumoniae*. Hierarchical cluster analysis was performed on signal values from 3 replicate experiments using CLUSFAVOR 6.0 and Spotfire DecisionSite 7.3 programs. **A**. CLUSFAVOR 6.0 graphical representation of a cluster representing a set of genes down-regulated under LSMMG conditions compared to 1 x g controls. Normalized intensity values are displayed on the y-axis and the x-axis represents each experiment. **B**. Heat map of a Spotfire DecisionSite 7.3 cluster showing a set of down-regulated genes under LSMMG conditions. Higher signal values are shown in red, lower signal values are green, and black represents median signal values.

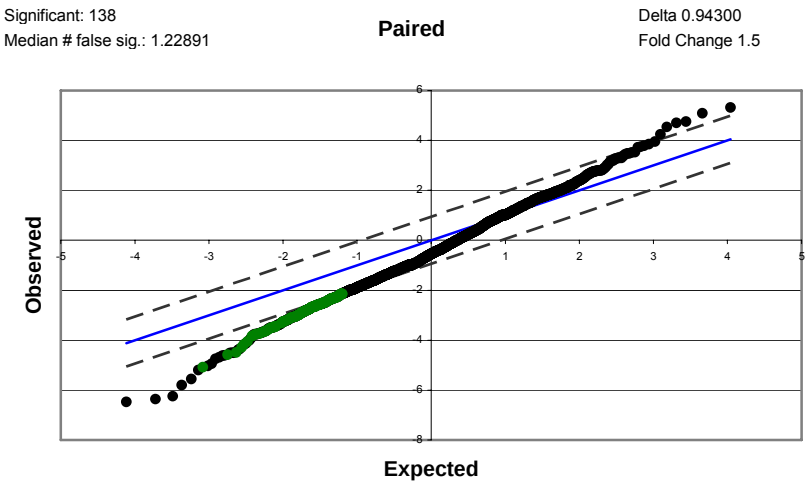
Table 3. Significance table showing delta values generated by SAM for *S. pneumoniae* grown under 1 x g vs. LSMMG conditions.

Analysis method	δ value	No. of Genes Called	No. of Genes Falsely Called	FDR
Paired				
	0.88	200	3.69	1.84%
	0.93	148	1.84	1.24%
	0.94	138	1.23	0.90%
	0.99	124	1.23	0.99%
	1.01	103	0.61	0.60%
Two-class				
unpaired,	0.18	250	46.5	18.6%
blocked	0.19	185	30.2	16.3%
	0.22	126	16.3	13.0%
	0.23	110	13.9	12.6%
	0.24	53	6.53	12.3%

δ values calculated by SAM using student t test
No. = Number
FDR = False Detection Ratio [(no. genes falsely called)/(no. genes called)]

blocked comparisons to generate fold-changes and *q*-values (similar to *p*-value) for genes determined by the user to be significant, based on an adjustable delta value which corresponds to an assigned false positive rate (Table 3). The advantage of SAM is that it allows the user to determine the stringency of the statistical analysis by choosing a delta value which will allow the number of significantly altered genes to be adjusted based on a corresponding FDR (expressed as a percentage). The delta value chosen for the two comparisons carried out allowed for the largest number of significant genes with the lowest false detection ratio assigned. Using a paired analysis parameter, a delta value of 0.943 and a minimum fold-change threshold of 1.5 produced 138 significant genes with an FDR of 0.90% for 1 x g vs. LSMMG conditions (Table 3, Fig. 5A). A two-class unpaired, blocked comparison parameter yielded similar results using a delta value of 0.215 and a 1.5 fold-change threshold generating 126 significantly altered genes with an FDR of 13.0 % (Table 3, Fig. 5B). These correlating results indicate that regardless of the comparison approach selected, SAM successfully identified significantly altered

A.



B.

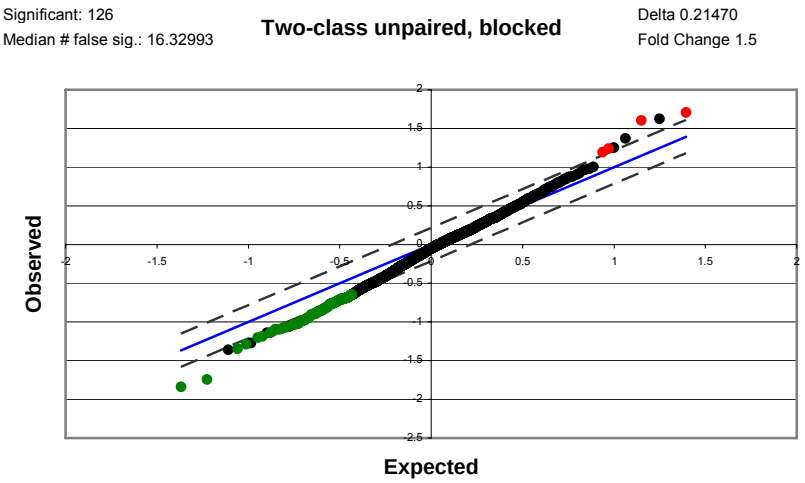


Fig. 5. Significance Analysis of Microarrays (SAM) plots for 1 x g vs. LSMMG-grown *S. pneumoniae*. SAM was used to compare signal values for *S. pneumoniae* grown under 1 x g and LSMMG conditions from each replicate (6 arrays). Data were analyzed in Excel using the paired (**A**) and two-class unpaired, blocked (**B**) options, with the additional requirement of at least a 1.5-fold change in gene expression. **A**. The delta value (0.943) chosen yielded the greatest number of significant genes (138) with the lowest false detection ratio (1.2/138 = 0.90%). **B**. The delta value (0.215) chosen yielded 126 significant genes with a false detection ratio of 13.0% (16.3/126). Significantly up-regulated genes are shown in red, and significantly down-regulated genes are shown in green.

LSMMG-responsive genes with modest changes in the FDR. The trend of down-regulated transcriptional activity in response to LSMMG conditions was observed for all analysis techniques utilized. In addition to the 1 x g rotating samples, cells grown under static gravitational conditions were included to serve as secondary negative controls. Any genes found to be differentially expressed between 1 x g rotating and static gravitational controls represented variations that were not directly attributed to the LSMMG environment and were thus excluded from further analyses.

Global transcriptional profile of *S. pneumoniae* in response to LSMMG compared to 1 x g control conditions.

The stringent 4-method analysis used identified only down-regulated genes in bacteria grown under LSMMG conditions, compared to 1 x g control cultures. Microarray analysis of *S. pneumoniae* cultures grown under LSMMG revealed the differential expression of 81 genes, all of which were found to be down-regulated in comparison to 1 x g control cultures (Table 4). LSMMG-responsive genes represented a wide range of diverse functional categories, in addition to uncharacterized genes. The differential expression of these various genes indicates that growth of *S. pneumoniae* under LSMMG can affect many different physiological functions, including metabolism, signaling, DNA repair/recombination, surface protein expression, and ion transport. These differentially expressed genes were physically located throughout the genome, which suggests that LSMMG conditions impact global transcriptional activity encompassing the entire chromosome. There were, however, 17 genes identified by microarray analysis that were up-regulated based on fold-change alone, but that did not meet the additional threshold criteria established for significance. Most of these up-regulated genes were found to be uncharacterized, but included the following characterized genes: 3 ATP transporters (*SP0092*, *SP0600*-Vexp2 ATP-binding protein, *SP0912*), 1 pyrimidine-nucleoside phosphorylase (*SP0842*), and 1 transcriptional regulator (*SP0246*, DeoR family). DeoR and DeoR homologs have been shown to function as transcriptional repressors for operons involved in deoxynucleoside catabolism (24, 150).

Microarray results were independently confirmed by measuring gene expression changes of seven down-regulated genes randomly selected and analyzed by quantitative real-time RT-PCR (Table 5). The real-time RT-PCR results confirmed decreased expression of all 7 genes, in concordance with the microarray results.

Table 4. Functional categories of *Streptococcus pneumoniae* genes differentially expressed under LSMMG

Locus	Putative encoded function	Gene Name	GenePix	Spotfire	SAM	ANOVA[a]
			Fold Change			***p value***
Antibiotic resistance						
SP1985	dimethyladenosine transferase	*ksgA*	-27.7	-15.4	-4	0.02
Cell envelope						
SP1833	cell wall surface anchor family protein		-48.5	-27.2	-2.3	0.03
SP2052	competence protein CglB	*cglB*	-26.6	-15.1	-2.9	0.02
SP2051	competence protein CglC	*cglC*	-6.8	-3.5	-1.8	0.04
SP1770	glycosyl transferase, family 8		-56	-31.6	-2	0.04
SP0536	immunity protein BlpL	*blpL*	-6.1	-3.1	-1.7	0.05
SP0544	immunity protein BlpX		-44.2	-31.6	-1.6	0.03
SP0545	immunity protein BlpY		-25	-13.9	-2.2	0.01
SP2063	LysM domain protein, authentic frameshift		-60.6	-43	-2.5	0.01
SP0637	membrane protein		-27.9	-19.6	-3.3	0.05
SP1823	MgtC/SapB family protein		-21.7	-12	-2.6	0.01
SP1867	NAD-dependent epimerase/dehydratase family protein		-3.3	-1.8	-2.2	0.01
DNA repair/recombination						
SP0575	helicase, putative		-21.2	-11.7	-2.6	0.01
SP0890	integrase/recombinase, phage integrase family		-20.4	-11.6	-1.7	0.01
SP1052	phosphoesterase, putative		-132.5	-94.4	-11	< 0.01
SP1902	Similar to regulatory protein recX	*recX*	-4	-2.2	-2	0.01
Metabolism						
SP0586	5,10-methylenetetrahydrofolate reductase, putative		-24.1	-13.3	-1.9	0.03

SP0566	acetyltransferase, GNAT family		-43.3	-24.6	-1.6	0.02
SP0113	argininosuccinate synthase, truncation	*argG*	-23.9	-16.8	-5.7	0.01
SP0964	dihydroorotate dehydrogenase B	*pyrDb*	-2.5	-1.6	-1.6	0.03
SP0251	formate acetyltransferase, putative		-34.8	-19.5	-1.8	0.01
SP1854	galactose operon repressor	*galR*	-18.2	-10.3	-1.8	0.01
SP1814	indole-3-glycerol phosphate synthase	*trpC*	-21.3	-11.8	-3.1	0.01
SP0321	PTS system, IIA component		-25	-14.2	-1.7	0.01
SP0305	PTS system, IIB component		-8.1	-4.5	-1.6	0.04
SP0061	PTS system, IIB component		-49.4	-35	-2.1	0.02
SP0062	PTS system, IIC component		-12.1	-6.8	-3.7	0.01
SP0647	PTS system, IIC component, putative		-19	-16.1	-2.4	0.01
SP1214	transulfuration enzyme family protein, authentic point mutation		-31.9	-17.8	-13.5	0.01
Signaling						
SP1784	hypothetical protein		-10.3	-5.4	-1.9	0.02
SP1096	putative adenylate cyclase		-78	-44.1	-4.4	0.02
SP1201	serine/threonine protein phosphatase		-21.4	-11.7	-2.2	0.02
Stress response						
SP1996	universal stress protein family	*uspA*	-4.7	-2.6	-2.3	0.03
Transcriptional/Translational regulation						
SP0120	glucose-inhibited division protein A	*gidA*	-54.6	-30.8	-2	< 0.01
SP0553	N utilization substance protein A	*nusA*	-28.8	-16.4	-1.8	0.01
SP0141	transcriptional regulator		-3.9	-2.4	-1.6	0.01
SP0395	transcriptional regulator, putative		-26	-14.4	-10.9	0.01
SP1423	transcriptional repressor, putative		-31.9	-17.8	-3.1	0.01
SP0171	ROK family protein		-47.3	-27.2	-1.6	0.01
Transporters/Ion channels						
SP0137	ABC transporter, ATP-binding protein		-44.2	-25.2	-1.9	0.03
SP1526	ABC transporter, ATP-binding protein authentic frameshift		-22.8	-12.9	-1.8	0.02
SP1357	ABC transporter, ATP-binding/permease protein		-36.6	-20.5	-4.4	< 0.01
SP0823	amino acid ABC transporter,		-45.5	-25.9	-4.6	0.01

	permease protein					
SP0729	cation-transporting ATPase, E1-E2 family		-2.2	-1.7	-2.3	0.05
SP0241	iron ABC transporter, permease protein, degenerate		-16	-11.1	-2.3	0.01
SP0408	sodium:alanine symporter family protein		-49.7	-28.6	-1.8	0.01
SP1318	v-type sodium ATP synthase, subunit G	*ntpG*	-29.5	-16.4	-1.7	< 0.01
Other						
SP0339	group II intron, maturase, truncation		-16.1	-8.8	-1.7	0.02
SP0315	IS200S, transposase, interruption		-44.7	-25.5	-1.5	0.01
SP1613	IS3-Spn1, transposase, authentic point mutation		-19.7	-10.8	-2.4	0.03
SP1402	NOL1/NOP2/sun family protein		-17.3	-9.4	-1.9	0.05
SP1916	PAP2 family protein		-16.8	-11.7	-2.9	< 0.01
SP1497	transposase, IS630-Spn1 related, Orf1, authentic frameshift		-6.5	-3.3	-1.7	0.04
SP0182	MccC family protein		-8.8	-4.9	-1.5	0.02
Unknown						
SP0723	conserved domain protein		-40	-28.3	-3.7	0.01
SP1250	conserved domain protein		-21.8	-11.9	-1.5	0.03
SP1437	conserved domain protein		-41.2	-23.1	-23.7	< 0.01
SP2071	conserved domain protein		-43.6	-24.5	-2.6	0.01
SP0379	conserved hypothetical protein		-19.7	-13.8	-1.8	0.02
SP0638	conserved hypothetical protein		-29.6	-20.8	-4.1	0.01
SP0686	conserved hypothetical protein		-15.1	-8.4	-1.5	0.01
SP0721	conserved hypothetical protein		-16.5	-9.6	-1.6	0.05
SP1327	conserved hypothetical protein		-13.9	-7.5	-2.6	0.03
SP1995	conserved hypothetical protein		-21.9	-12.4	-1.8	0.01
SP0116	hypothetical protein		-11.1	-5.9	-2.1	0.04
SP0167	hypothetical protein		-19.3	-13.5	-6.3	< 0.01
SP0297	hypothetical protein		-15.6	-8.8	-1.6	0.02
SP0826	hypothetical protein		-6.8	-4.9	-1.8	0.02
SP0924	hypothetical protein		-27.1	-15.7	-1.6	0.01
SP1078	hypothetical protein		-42.2	-23.7	-1.8	0.04
SP1562	hypothetical protein		-40.4	-22.6	-1.9	0.02

SP1635	hypothetical protein		-12.2	-6.6	-2	0.02
SP1677	hypothetical protein		-49.5	-35.1	-51	0.02
SP1952	hypothetical protein		-43.9	-24.7	-6.2	< 0.01
SP1955	hypothetical protein		-21.3	-12.1	-1.7	0.01
SP2120	hypothetical protein		-46.2	-26.3	-2.1	0.02
SP0031	hypothetical protein		-42.2	-24	-1.8	0.02
SP0068	hypothetical protein		-17.9	-9.8	-1.8	0.01
SP1065	hypothetical protein		-19.7	-10.8	-2.5	0.02
SP0331	hypothetical protein, authentic frameshift		-38.6	-22.3	-1.7	< 0.01
SP1931	hypothetical protein, fusion		-25.5	-14.1	-2.3	0.02

[a]ANOVA values calculated from 1 x g and LSMMG gene intensity values.

Table 5. Real-time RT-PCR confirmation of representative genes down-regulated under LSMMG conditions in *S. pneumoniae* using microarrays

Locus	Gene name	Microarray *	Real-time RT-PCR
		Fold Change	
SP0553	NusA	-1.8	-5.1
SP0575	Helicase, putative	-2.6	-12.2
SP1985	KsgA	-4.0	-3.0
SP1996	Universal stress protein, UspA	-2.3	-17.8
SP0241	ABC transporter	-2.3	-12.8
SP1096	Adenylate cyclase, putative	-4.4	-53.6
SP0545	Immunity protein BlpY	-2.2	-13.0

*Fold change based on SAM analysis

LSMMG-responsive down-regulated genes of common operons and/or clusters included members involved in competence, antimicrobial peptide production, and carbohydrate transport. Competence is a natural process in *S. pneumoniae* where the bacteria take on a transient physiological state which allows them to bind and take up exogenous DNA from the environment and then integrate it into their chromosome. The induction of competence occurs through a quorum-sensing process involving the

secretion of competence-stimulating peptide (CSP) to threshold concentrations of about 10 ng ml^{-1}, which then leads to the activation of the two-component system ComDE. Phosphorylation of ComE (response regulator) and ComD (histidine kinase) induces the activation of the alternative sigma factor ComX, which, in turn, initiates the transcription of several operons containing genes encoding a number of factors involved in DNA uptake, processing, and recombination (71). Two down-regulated genes (*cglB* and *cglC*) identified are members of the *cgl* operon, which is one of the late competence operons induced during the onset of competence in the *S. pneumoniae* life cycle (75, 106). The proteins encoded by these genes are involved in construction of the surface-expressed translocasome (DNA uptake apparatus) that functions in DNA uptake and processing during competence (75).

The microarray results revealed the down-regulation of three genes (*blpL*, *blpX*, and *blpY*) which are members of the *blp* (bacteriocin-like peptide) regulon, another quorum-sensing system which functions in the production of antimicrobial peptides and immunity proteins which are co-transcribed for intra- and interspecies competition during colonization (32, 33). Induction of bacteriocin production occurs through the detection of bacteriocin-inducing peptide (BIP) which activates the two-component system BlpRH and the subsequent activation of operons containing genes encoding bacteriocin structural and immunity proteins (32). Bactericidal activity of bacteriocins generally involves receptor-mediated mechanisms such as pore formation or membrane permeabilization. Producer strains of bacteriocins are protected through the synchronous production of immunity proteins which associate with the target surface receptors preventing pore formation (98). The *blpL*, *blpX*, and *blpY* genes are members of operons which encode the protective immunity proteins.

Knutsen *et al.* previously reported that the *qsrAB* operon, which encodes an uncharacterized ABC transporter, can undergo cross-induction by either of the two signaling pheromones (CPS or BIP) associated with the ComABCDE and BlpABCSRH two-component signaling pathways, respectively, due to the presence of a hybrid direct repeat motif which can recognize both response regulators (ComE and BlpR) (71). These findings indicate the potential for cross-talk between these two independent quorum-

sensing systems which both contain genes responsive to the LSMMG environment. Such responses may suggest that while the impact of LSMMG can affect many different operons and gene clusters of various functions throughout the genome, some of these groups could respond in a coordinated manner by interactions between pathways through convergent regulatory mechanisms.

Another group of down-regulated genes included five members (*SP0321*-PTS IIA, *SP0305*-PTS IIB, *SP0061*-PTS IIB, *SP0062*-PTS IIC, and *SP0647*-PTS IIC) of the phosphotransferase systems (PTS), involved in carbohydrate uptake and metabolism. The PTS system is generally composed of three-to-four functional components, IIA, IIB, IIC, and IID which are generated as either individual subunits in a complex or as different domains in a single polypeptide (e.g. IIAB). The first enzyme (EI) in the PTS cascade acquires a phosphoryl group from phosphophenol pyruvate (PEP), resulting in the dephosphorylation of PEP to pyruvate. The phosphotransfer chain continues as EI phosphorylates histidine-containing PTS phosphotransferase (HPr), followed by IIA, IIB, and finally IIC / IID, which contain sugar binding sites allowing phosphorylation and uptake of different carbohydrates from the environment (124). Diep *et al*. recently reported that the IICD membrane surface complex of the mannose PTS pathway can also serve as a target receptor for class II bacteriocins and corresponding immunity proteins (38). Thus, the down-regulation of *SP0062* and *SP0647* which encode the PTS IIC components may serve as a survival strategy during colonization in low-shear areas of the host to compete against other species. The genes reported above were found to exhibit changes in expression at levels above the established threshold for significance of additional genes within the competence. Additional members of the Blp and PTS regulons were also found to be down-regulated, albeit at levels below the threshold limits. These results suggest that LSMMG conditions generate an environment in which *S. pneumoniae* phenotypic changes may be characterized by decreases in DNA transformability, carbohydrate uptake, and the production of antimicrobial peptide/immunity peptides.

While most of the remaining down-regulated genes do not share common operons or clusters in the genome, several are functionally tied with the acquisition of antibiotic

resistance, stress resistance, and virulence. One of these genes encodes the rRNA adenine dimethyltransferase, KsgA, which catalyzes the methylation of 16S rRNA (102). Dimethylation of two adenosine bases in 16S rRNA is conserved across all prokaryotic species, and inactivation of the *ksgA* gene has been shown to lead to bacterial resistance to aminoglycoside antibiotics (e.g. kasugamycin) due to the loss of this modification. Alterations in the gene expression of antibiotic resistant genes, such as *ksgA*, could contribute to the trend of increased antibiotic resistance demonstrated by bacteria during space flight (93). The gene encoding universal stress protein A (UspA) was also found to be down-regulated under LSMMG. UspA is produced in response to a variety of internal and external stresses and is thought to be a member of the RecA-dependent DNA protection and repair system involved in the protection of DNA from UV damage (39). Liu *et al.* recently reported that UspA of *S.* Typhimurium LT2 plays an important role in conferring resistance to metabolic and oxidative stresses and also contributes to bacterial virulence in mice (82). The down-regulation of *uspA* and other stress-related genes could explain the increased sensitivity found in *S. pneumoniae* upon exposure to thermal and acid stresses after growth under LSMMG conditions (2). Two other genes that were found to be down-regulated under LSMMG conditions (*SP1823* and a homolog of *gidA*) are associated with virulence acquisition and regulation. *SP1823* encodes an MgtC/SapB family protein which has been demonstrated to function as a horizontally-acquired virulence factor responsible for survival in macrophages, virulence in mice, and growth under low Mg^{2+} conditions (12). The virulence regulator *gidA* (glucose-inhibited division protein) mediates the expression of cytotoxic enterotoxin and subsequent virulence in mice in the food-borne pathogen *Aeromonas hydrophila* (123). The repression of certain virulence genes by bacteria grown under LSMMG may play an important functional role in immune evasion, thus contributing to their survival and pathogenesis.

Previous studies in the laboratory of Dr. David Niesel have shown that *S. pneumoniae* exhibits an increased ability to adhere to and invade A549 respiratory epithelia after growth under LSMMG conditions (unpublished data). Our microarray analysis revealed that the genes encoding the various factors associated with these processes were not significantly affected by growth under LSMMG conditions. Thus, no

direct correlations were identified which could explain the altered physiological properties observed. A similar trend was observed for *S.* Typhimurium strain χ3339 after growth under LSMMG. While virulence, survival in macrophages, and stress resistance were shown to be enhanced after growth under LSMMG, none of the genes encoding known virulence factors were induced after growth in LSMMG (99, 147, 148). A correlation was observed between the down-regulated genes involved in LPS biosynthesis and LPS protein expression. SDS-PAGE analysis of LPS levels correlated with array findings, indicating a decrease in protein expression compared to 1 x g control cultures. While microarray analysis of strain χ3339 indicated no enhancement of virulence gene expression, other reports have demonstrated increases in virulence and environmental stress resistance by other bacterial strains and species after growth under LSMMG (99, 147). LSMMG-grown *S.* Typhimurium strain 14028 has been shown to demonstrate increased invasion potential, stimulate TNF-α production in infected epithelia, and exhibit altered gene and protein expression (23). Microarray analysis of strain 14028 showed a trend similar to that of χ3339, in that most of the differentially expressed genes were down-regulated in response to LSMMG; however one gene encoding a virulence factor, VirK (involved in membrane remodeling and motility), was found to be induced, which might suggest strain-specific differences in response to this environment.

In a recent study evaluating changes in the genomic profile of *E. coli* MG1655 after growth under LSMMG, Wilson *et al*. reported differential gene expression in cultures grown in both rich media (LB) and minimal media (MOPS) (142). Results from this study indicated that the majority of LSMMG-responsive genes were down-regulated in cultures grown in both media and that the majority of these genes did not correlate to those found to be responsive to LSMMG in strain χ3339. LSMMG-responsive genes (16 up-regulated, 19 down-regulated) from MOP cultures included the induction of genes associated with chemotaxis, flagella synthesis, and acid stress tolerance while genes involved in stress responses, transport, and envelope function were down-regulated. Transcriptional responses in LB cultures (1 up-regulated, 14 down-regulated) included

the induction of *accA* (encodes carboxyl transferase subunit) and the down-regulation of genes involved in biosynthesis, energy utilization, and ribosomal genes.

Chapter 5: The Effects of Low-Shear Modeled Microgravity on Adherent-Invasive *Escherichia coli* Physiology and Colonization Potential

INTRODUCTION

Clinical isolates from Crohn's Disease (CD) patients have been categorized in a new putative class of *E. coli* known as adherent-invasive *E. coli* (AIEC) based on their unique properties which allow these strains to play a contributory role to the chronic inflammatory state representative of the pathology of inflammatory bowel disease (29). There is currently a limited amount of information on the factors and regulatory mechanisms used by these strains to adhere to and invade host cells and stimulate cytokine production. Additionally, no known virulence factors have been identified which could classify these isolates in one of the major classes of pathogenic *E. coli*. In an effort to understand how these strains respond and adapt to the environmental factors encountered in the gut, HARVs were used as a model system to cultivate AIEC strain O83:H1 in a low-shear, low-turbulence environment, as experienced in the gastrointestinal tract. While previous reports have shown non-pathogenic commensal strains of *E. coli* to undergo alterations in stress resistance and gene expression in response to LSMMG, no clinical isolates such as AIEC isolates have been studied to understand how mechanical stresses affect their physiology (85, 142). Reports have shown conflicting evidence regarding a role for the global stress response regulator RpoS in different enteric species grown under LSMMG, and nothing is known regarding this regulator in clinical isolates grown in this unique environment (85, 147). Therefore, an *rpoS* isogenic mutant was generated from AIEC O83:H1 to evaluate the putative role of this global regulator in AIEC clinical isolates in response to the LSMMG environment.

RESULTS AND DISCUSSION

The impact of LSMMG on AIEC O83:H1 growth kinetics and RpoS protein expression.

The impact of LSMMG on the growth kinetics of AIEC O83:H1 and *rpoS* strain CAA001 was examined by monitoring their growth in M9 minimal media within HARVs oriented in either LSMMG or 1 x g control positions for an overall period of 12 hours. A defined M9 minimal media was selected for growing the bacterial cultures in the HARVs instead of a complex rich media such as LB. Bacterial cultures were grown for 12 hours to ensure cultures had entered stationary phase in which RpoS expression and activity occur at maximal levels. Twenty-four hour cultures (late-stationary phase) were avoided to rule out any starvation effects due to media depletion which could impact experimental results. Samples taken every two hours exhibited a similar cell density and equivalent bacterial counts from both LSMMG and 1 x g control cultures. For example, at 12 hours, bacterial counts were as follows: AIEC O83:H1, 2.6 x 10^9 CFU (1 x g) and 2.5 x 10^9 CFU (LSMMG); CAA001, 1.5 x 10^9 CFU (1 x g) and 1.7 x 10^9 CFU (LSMMG). We included *E. coli* K-12 strain AMS6, previously examined under LSMMG by Lynch *et al.* (85), as a bacterial control which exhibited bacterial counts of 7.3 x 10^8 CFU, in 1 x g, and 7.0 x 10^8 CFU, in LSMMG. Similar growth correlations using AMS6 under LSMMG have previously been reported, which support the validity of our experimental conditions. Overall, only slight increases in growth were observed in CAA001 compared to the wild-type strain regardless of the culture condition tested (Fig. 6). These data exclude the possibility of growth-dependent variations as an independent variable impacting experimental analyses. Growth phase-dependent alterations in RpoS protein expression, such as reduced and increased expression in exponential and late stationary phases, respectively, have been observed in *E. coli* AMS6 under LSMMG conditions (85). Therefore, RpoS expression in AIEC O83:H1 was investigated to determine if this trend in expression holds true using a clinical AIEC isolate grown under conditions of LSMMG. AIEC O83:H1 RpoS protein levels were shown to increase over time; however, increases in expression were found to be independent of growth under LSMMG versus 1 x g controls in contrast to previous findings reported by Lynch *et al.* (Fig. 7) (85). The Western blot analysis results shown are representative of exponential (4 hrs) and stationary phases (12 hrs) of growth correlating with growth kinetic data. Our

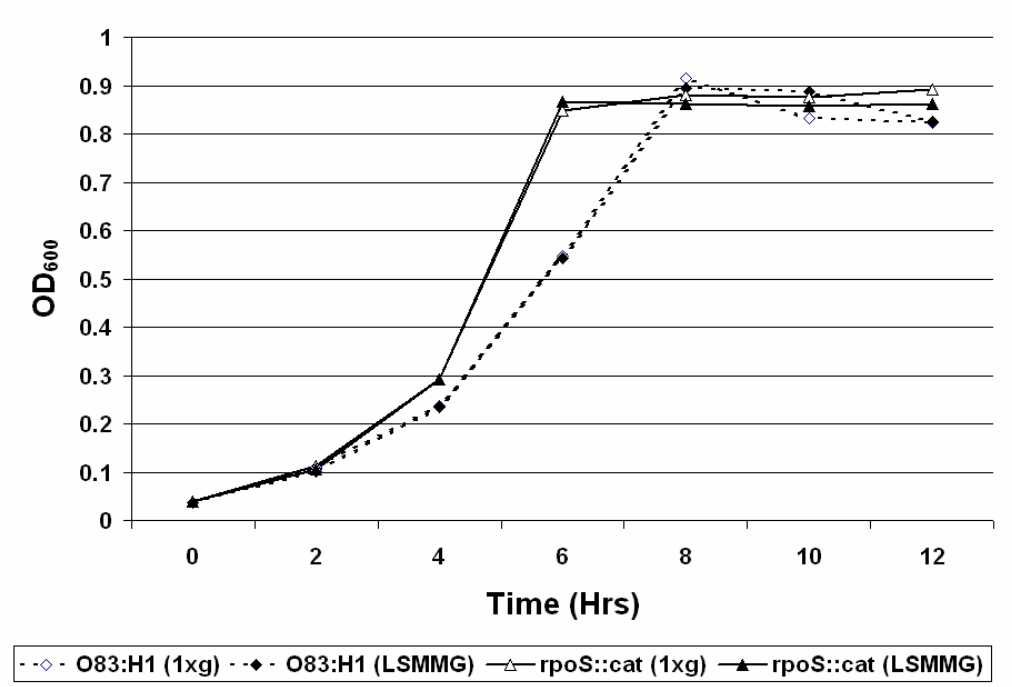


Fig. 6. Growth kinetics of AIEC O83:H1 and *rpoS* mutant strain CAA001 grown under LSMMG. Overnight cultures of AIEC O83:H1 and CAA001 were diluted 1:100 in fresh M9 minimal media and incubated in HARVs positioned for LSMMG and 1 x g conditions. Culture growth rate and quantity were monitored by measuring optical densities at 600 nm. Data presented are representative of results from two independent trials, all of which reflect comparable growth characteristics.

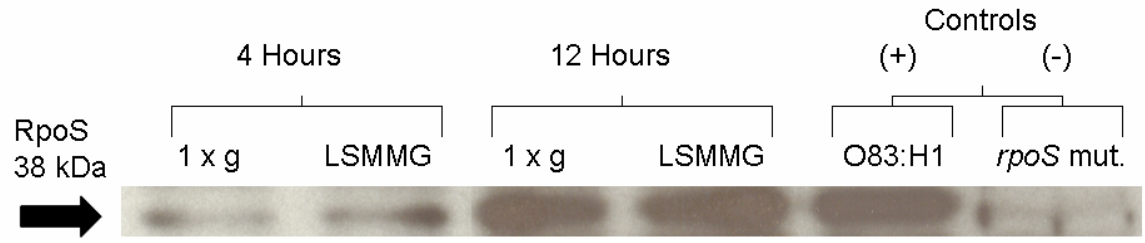


Fig. 7. RpoS protein expression is not altered under LSMMG. Western blot analysis revealed RpoS expression to increase over time (4 and 12 hours); however the increased expression was independent of growth under LSMMG conditions. Controls included wild-type AIEC O83:H1 (+) and *rpoS* mutant CAA001 (-) test tube cultures.

findings at late stationary phases of growth (24 hrs) are similar to the increased RpoS expression observed at the 12 hr time point.

Results from our growth kinetics analysis of AIEC O83:H1 and the *rpoS* mutant CAA001 grown in M9 minimal media under LSMMG conditions indicate that the mutant strain enters exponential phase and reaches stationary phase at slightly faster rates when compared to the wild-type strain (Fig. 6). This increased growth was found to be independent of the LSMMG environment since each strain was found to grow at similar rates when LSMMG and 1 x g cultures were compared. Lynch *et al.* reported that no major differences were observed in wild-type *E. coli* AMS6 grown in M9 minimal media under LSMMG or 1 x g controls; however, no information was given regarding changes in growth rates by an isogenic *rpoS* mutant generated from the parental strain (85). The growth kinetics of *E. coli* MG1655 were also previously evaluated in HARVS in both LB and MOPS minimal media (142). In this study the lag phase of MOPS cultures was longer than that of LB cultures irrespective of LSMMG and 1 x g control orientations. In addition, exponential cultures grown in MOPS were found to grow at similar rates in LSMMG and 1 x g control HARVs. This is in contrast to LB exponential cultures, which exhibited slower growth under LSMMG compared to 1 x g conditions. Wilson *et al.* demonstrated that wild-type *S.* Typhimurium strain χ3339 and an isogenic *rpoS* mutant showed increased growth kinetics under LSMMG conditions compared to 1 x g control cultures when grown in M9 minimal media. These effects were not observed when the bacterial strains were grown in Lennox Broth (rich media) (147). These findings suggest that the effects of LSMMG in combination with growth in minimal media can enhance the growth kinetics in certain strains and species of bacteria, though the effect is not universal. Environmental stresses such as low fluid shear and nutritional limitations correspond to the natural conditions enteric species encounter in the gut. In addition, RpoS does not appear to have a significant impact on the growth of AIEC O83:H1 and *S.* Typhimurium under these conditions, based on our findings and those reported by Wilson *et al*., since diminished growth rates were not observed in the mutant strains. It remains to be determined however, if this observation holds true for commensal strains of *E. coli* such as AMS6 and MG1655.

Lynch *et al.* previously reported that RpoS protein expression was increased in stationary cultures of *E. coli* AMS6 grown under LSMMG and that responses to certain stresses were RpoS-dependent during this growth phase. We, therefore, evaluated RpoS expression in our clinical isolate to determine if this trend was conserved across different strains. While RpoS protein expression was found to increase over time, as previously established, this increased expression occurred in both LSMMG and 1 x g control cultures, suggesting that expression changes are independent of the LSMMG environment in AIEC O83:H1 (Fig. 7) (68). These findings suggest that any potential RpoS-mediated effects on AIEC O83:H1 under LSMMG conditions do not occur primarily due to changes in protein expression levels, but possibly at other levels of regulation (e.g. transcriptional level).

Altered environmental stress resistance in AIEC O83:H1 and *rpoS* strain CAA001 under LSMMG.

AIEC O83:H1 and CAA001 were next examined to determine if growth under LSMMG conditions altered survival to different environmental stresses compared to growth under 1 x g controls. AIEC O83:H1 was evaluated for changes in resistance to thermal, osmotic, acid, and oxidative stresses after growth under LSMMG to determine whether this environment can alter the response of AIEC to different environmental stresses. AIEC O83:H1 exhibited a significant increase in thermal stress resistance (Fig. 8A) after growth under LSMMG conditions compared to 1 x g control cultures. Enhanced thermal stress resistance has also been observed in *Salmonella* after growth under LSMMG (147). Increased resistance was also observed after exposure of AIEC O83:H1 to oxidative stress for 30 minutes (Fig. 8B). While there appeared to be a slight decrease in resistance to osmotic stress by LSMMG-grown AIEC O83:H1, no significant differences were observed between LSMMG and 1 x g cultures in response to acid stress (Figs. 8C and D).

E. coli strain AMS6 was included in the stress assays to function as an internal bacterial control. The purpose of including this strain was to show that the use of the

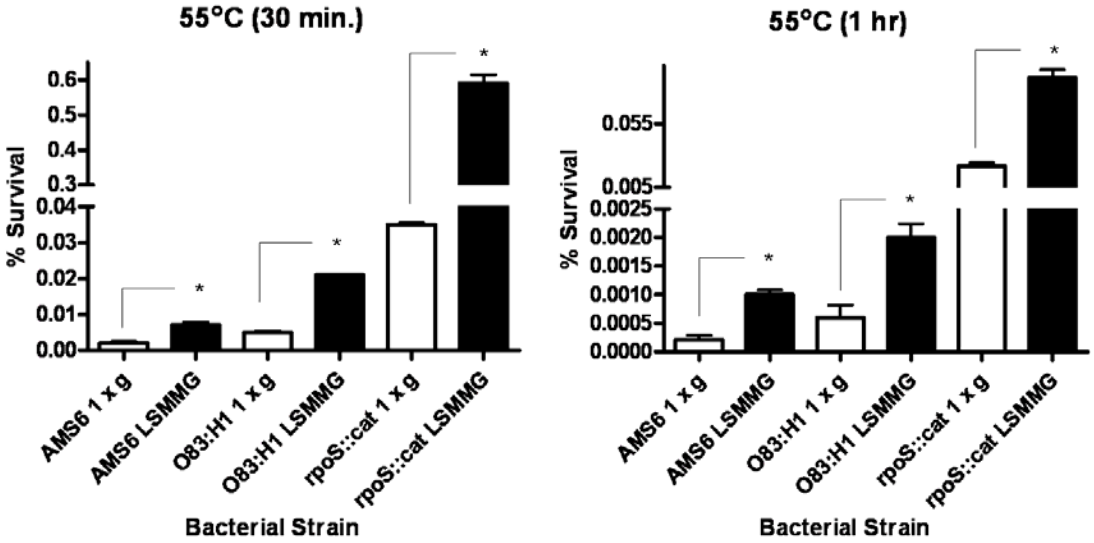
HARV system was comparable to that by other laboratories and, in doing so, similar trends in stress resistance (e.g. increased osmotic and acid stress resistance) were successfully reproduced as previously published with this same strain by Lynch *et al.* (Fig. 8) (85). In addition, the previous findings were expanded by including in the analyses thermal and oxidative stresses, which revealed significant increases in thermal stress resistance and oxidative stress sensitivity exhibited by the AMS6 strain after growth under LSMMG.

It was observed that both the AMS6 and AIEC O83:H1 strains exhibited an increased resistance to thermal stress (Fig. 8A), while displaying opposing trends in resistance to osmotic and oxidative stresses (Fig. 8B and C). As previously reported, the AMS6 strain exhibited an increase in acid stress resistance after growth under LSMMG, while AIEC O83:H1 exhibited no major differences to this stressor regardless of the growth environment (Fig. 8D). It was observed that the increase in oxidative stress resistance by LSMMG-grown AIEC O83:H1 appeared to diminish between 30 minutes and 1 hour of exposure (Fig. 8B), which could indicate the reversion of LSMMG-induced phenotypic characteristics, whereas the resistance profiles observed during exposure to the other three stressors persisted throughout both challenge time points.

Examination of the *rpoS* mutant strain CAA001 showed that growth under LSMMG not only increased thermal stress resistance by the mutant, as observed in the LSMMG-grown wild-type AIEC and AMS6 strains, but the enhanced resistance was also at significantly higher levels (Fig. 8A). An increase in osmotic stress resistance was also exhibited by LSMMG-grown CAA001 as observed in strain AMS6 (Fig. 8C). In addition, both strains demonstrated a decreased resistance to oxidative stress after growth under LSMMG conditions though the sensitivity by CAA001 was much higher to this stressor (Fig. 8B). Neither CAA001 nor the wild-type strain exhibited LSMMG-dependent differences to acid stress conditions in contrast to the enhanced resistance exhibited by LSMMG-grown AMS6 (Fig. 8D).

Previous reports investigating *E. coli* strains AMS6 and MG1655 have indicated contrasting trends in stress resistance after growth under LSMMG conditions in minimal media. While the AMS6 strain was found to exhibit increased resistance to acid and

A.



B.

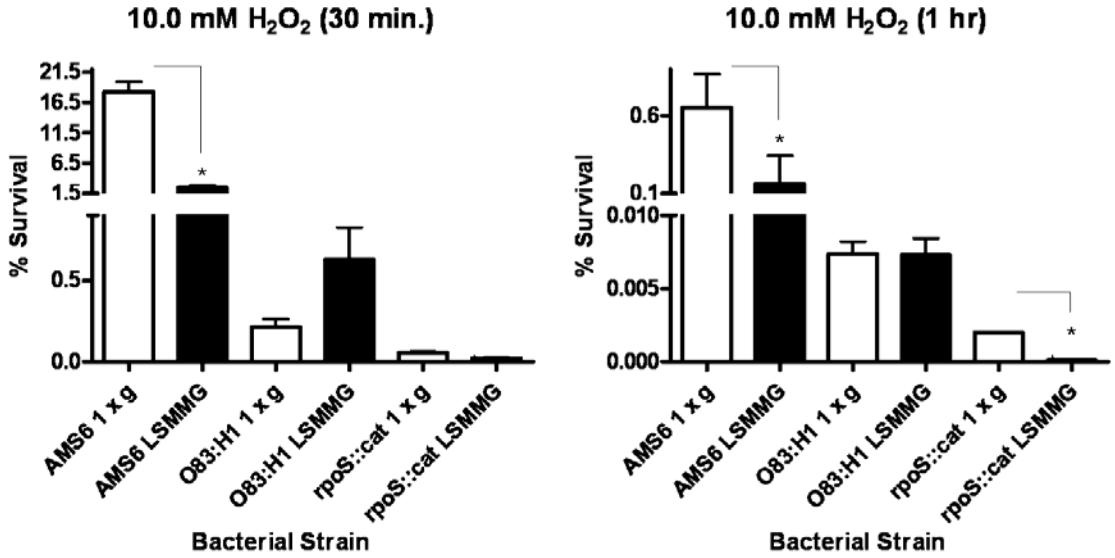
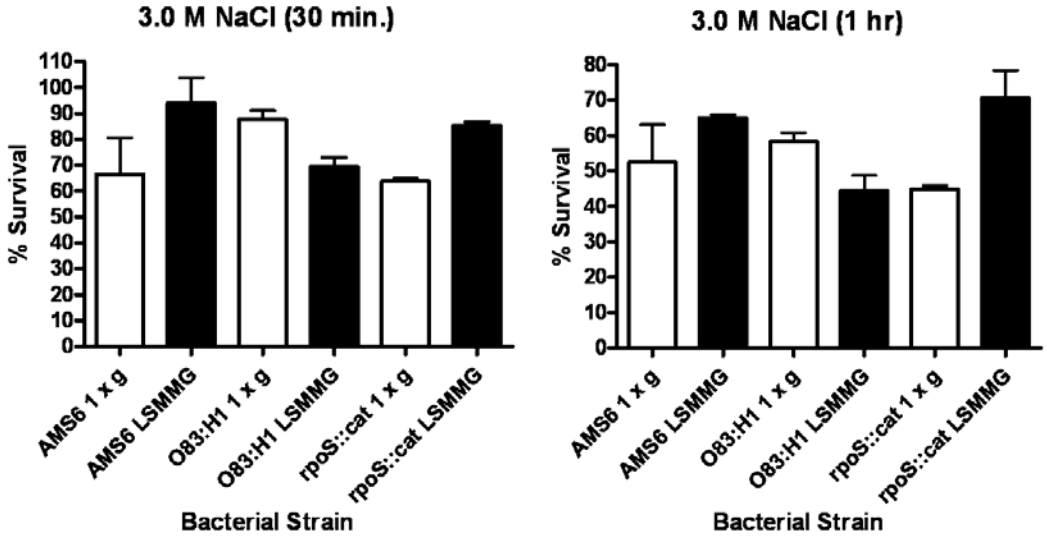


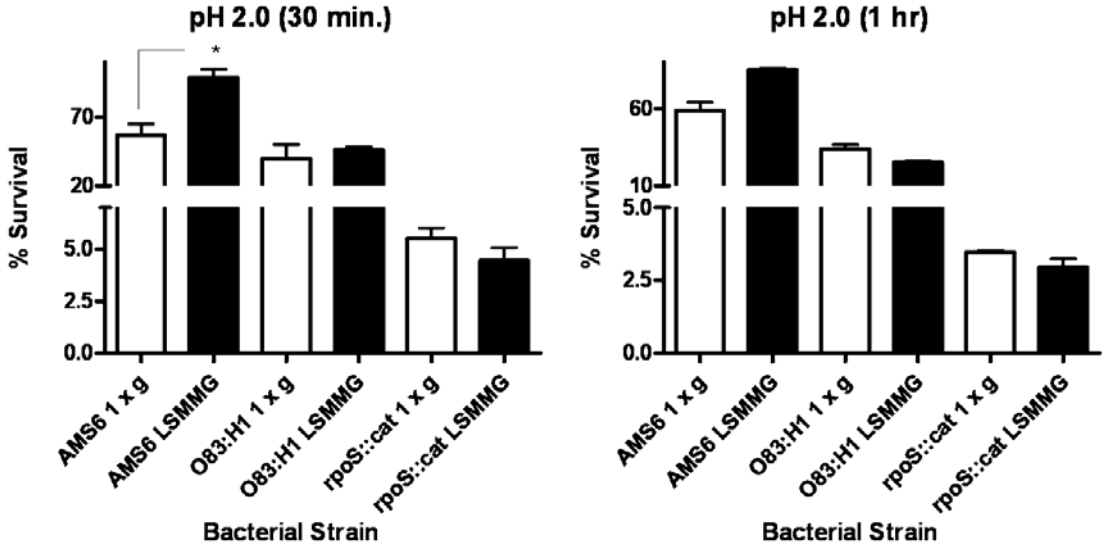
Fig. 8. Altered resistance to environmental stresses by LSMMG- grown AIEC O83:H1 and *rpoS* mutant (CAA001). Bacteria from LSMMG (■) and 1 x g control (□) cultures were exposed to either (A) thermal (55°C), (B) oxidative (10.0 mM H_2O_2), (C) osmotic (3.0 M NaCl), or (D) acid (pH 2.0) stress for 30 minute and 1hour at room temperature. The percentage of survival was calculated from plate counts prior to and after exposure periods. Data presented are representative of 3 independent trials. Error bars correspond to standard error of means and asterisks indicate statistical significance ($p \leq 0.05$).

(Fig. 8 continued)

C.



D.



osmotic stresses after growth under LSMMG, the MG1655 strain did not show any significant changes in stress resistance to not only these two stressors, but also towards additional stresses including alkaline, thermal, oxidative, and ethanol stresses (85, 142). The AIEC O83:H1 clinical isolate evaluated in this study demonstrated a combination of both increases in resistance and sensitivity to the different environmental stresses examined. In some of these cases, the effects appeared to be independent of growth in the LSMMG environment. Stress resistance was also previously investigated in the enteric pathogen *S.* Typhimurium after growth in minimal media under LSMMG conditions. Results from this study demonstrated that while growth under LSMMG resulted in a phenotype exhibiting enhanced resistance to thermal, osmotic, and acid stresses, sensitivity to oxidative stress was also increased (147). These results suggest that growth under conditions of LSMMG can alter bacterial physiology impacting the ability of the bacterium to resist different stresses and that this is not a universal effect. Furthermore, these changes not only vary between different bacterial genera, but also can alter bacterial physiology in a strain-dependent manner.

The impact of RpoS on stress resistance under LSMMG conditions has been shown to differ between different bacterial genera and in some cases also by growth phase. In *S.* Typhimurium and *E. coli* AMS6, environmental stress resistance was found to be independent of RpoS expression under LSMMG in stationary and exponential phase cultures, respectively (85, 147). In contrast, both acid and osmotic stress resistances were found to be diminished in LSMMG-stationary phase cultures of an *E. coli* AMS6 *rpoS* mutant (85, 147). Therefore, the impact of RpoS was evaluated in AIEC O83:H1 under LSMMG during stationary phases of growth where RpoS expression occurs at maximal levels (Fig. 7). Comparisons between CAA001 and the wild-type strain revealed similar trends in resistance to thermal (increased resistance) and acid (no change between LSMMG and 1 x g) stresses under LSMMG conditions (Fig. 8 A and D). In contrast, CAA001 exhibited slight increases in resistance to osmotic stress and decreased oxidative stress resistance after growth in LSMMG compared to the wild-type strain (Fig. 8B and C). While the presence of RpoS expression appears to be important for oxidative and

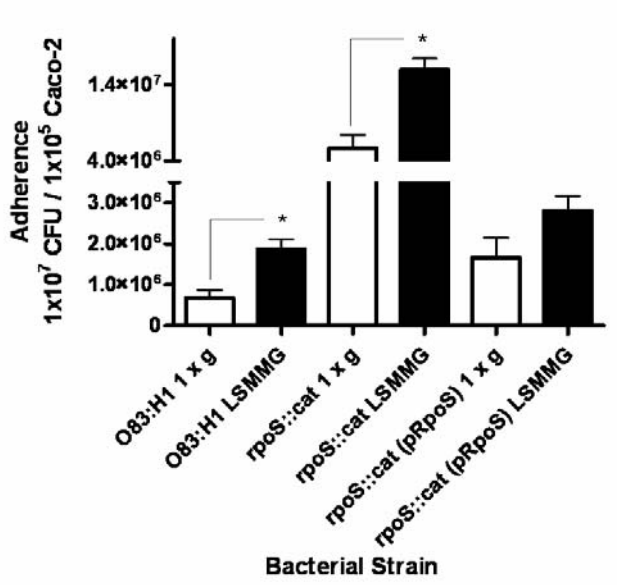
acid stress resistance under LSMMG, it is not clear as to how the absence in activity of this critical stress response regulator can lead to increased thermal and osmotic resistance in the LSMMG environment. Based on similar stress resistance patterns exhibited by both LSMMG-grown wild-type and *rpoS* mutant *S.* Typhimurium strains, Wilson *et al.* has suggested that environmental cues from the LSMMG environment may initiate RpoS-independent changes in the physiology of bacteria allowing them to phenotypically become pre-adapted to resist multiple environmental stresses (147). Perhaps cues from the LSMMG environment function to elicit a combination of both RpoS-dependent and independent effects in AIEC O83:H1 in a stress-specific manner. Alternatively, previous reports have shown varying degrees of heterogeneity in RpoS functionality and subsequent resistance toward different environmental stresses in different pathogenic strains of *E. coli* (10).

Collectively, these findings demonstrate that growth under LSMMG can alter bacterial resistance to different stresses and that these changes are not universal, but can vary between different genera and strains. Furthermore, results from assays with the CAA001 demonstrated an important role for RpoS in mediating protection against certain stresses (e.g. oxidative and acid stresses) under LSMMG conditions during stationary growth periods. The results presented here reveal, for the first time, the effects of LSMMG and the impact of RpoS on the ability of an AIEC clinical isolate to tolerate different environmental stresses.

Enhanced adherence by AIEC O83:H1 to Caco-2 cells after growth under LSMMG.

AIEC clinical isolates are characterized by their intrinsic ability to adhere to and invade gastrointestinal epithelial cells and residential macrophages, resulting in the production of TNF-α, which can contribute to pro-inflammatory responses (61). Therefore, we examined the impact of LSMMG on these two central characteristics using Caco-2 cells. Twelve-hour cultures of AIEC O83:H1 grown under LSMMG and 1 x g control conditions were used to infect Caco-2 monolayers. AIEC O83:H1 LSMMG cultures were found to exhibit significantly higher adherence levels to Caco-2

A.



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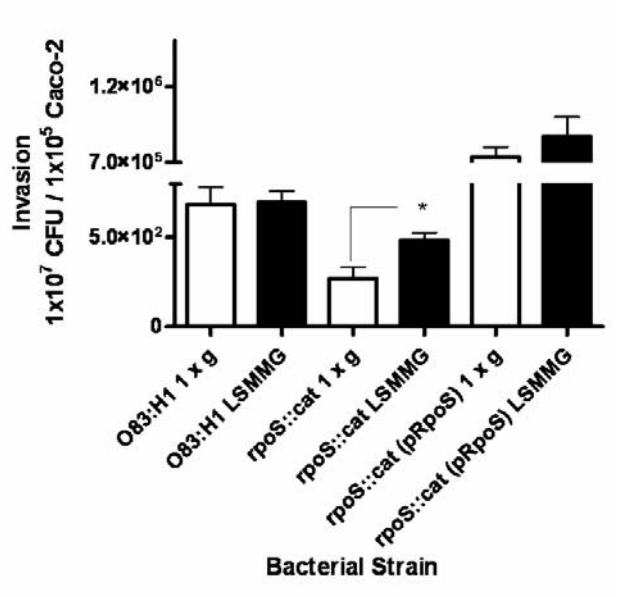


Fig. 9. Adherence and invasion of AIEC O83:H1 and *rpoS* mutant CAA001 to Caco-2 cells. Twelve-hour LSMMG (■) and 1 x g (□) cultures were used to infect Caco-2 monolayers (MOI of 100:1) for either (A) adhesion and (B) invasion assays. The *rpoS* mutation in CAA001 was complemented by introduction of pRpoS (containing an intact *rpoS* gene). Data are representative of 2 independent trials. Error bars correspond to standard error of means generated from triplicate wells. Asterisks indicate statistical significance ($p \leq 0.05$).

monolayers in comparison to the 1 x g cultures (Fig. 9A). In contrast, no significant differences were observed in Caco-2 invasion between LSMMG and the 1 x g cultures (Fig. 9B). Next, the *rpoS* mutant CAA001 was examined for LSMMG-induced changes in adherence and invasion. CAA001 displayed increases in both adherence and invasion capabilities after growth under LSMMG conditions (Fig. 9 A and B). Comparing changes in adherence between CAA001 and the wild-type strain under LSMMG conditions revealed that the levels of adherence demonstrated by CAA001 were 8.5-fold higher than those in LSMMG-grown AIEC O83:H1 cultures (Fig. 9A). While the phenotype of CAA001 under LSMMG demonstrated a greater ability to adhere to Caco-2 cells, comparisons between the mutant and wild-type strain suggested that the overall invasive ability of CAA001 was diminished in both the LSMMG and 1 x g control environments (Fig. 9B). To verify that the absence of RpoS expression was the impacting factor responsible for the alterations in adherence and invasion of CAA001 under LSMMG, CAA001 was complemented with pRpoS (containing an intact *rpoS* gene). The adherence phenotype exhibited after complementation of CAA001 correlated to those observed by the wild-type strain during growth under LSMMG and 1 x g control conditions (Fig. 9A). While complementation of CAA001 did show a reversion in the invasion phenotype, the levels of invasion observed were much higher than that of the wild-type strain (Fig. 9B). This increase could be attributed to an increase in the copy number of RpoS transcripts compared to those produced by the wild-type strain due to the fact that pGEMTEasy (a high copy plasmid) was used as the cloning vector to construct pRpoS for the complementation of CAA001 (Table 1).

The LSMMG environment has previously been shown to enhance the colonization and invasion potential of bacteria. Nickerson *et al.* has shown using a murine infection model that growth of *S.* Typhimurium under LSMMG conditions increases the ability of this pathogen to colonize different organs such as the spleen and liver upon oral challenge, in comparison to 1 x g control cultures (99). The invasion potential of *S.* Typhimurium has also been shown to be enhanced after growth in the LSMMG environment. Chopra *et al.* showed that invasion of both epithelial cells (SKOV3) and macrophages (RAW 264.7) was increased when infected with *S.*

Typhimurium cultures grown under LSMMG conditions compared to 1 x g controls (23). The findings presented here show that LSMMG can also enhance the colonization potential of clinical isolates of AIEC through physiological changes which increase the capabilities of such strains to adhere to intestinal epithelia.

While RpoS is well-known for its role as an important global stress regulator in response to various environmental stresses, it is less clear as to what regulatory role this sigma factor plays in the process of colonization and virulence potential. Bhagwat *et al.* has previously shown that there are various degrees of RpoS functional heterogeneity between different *E. coli* serotypes and strains regarding adherence (10). Complementation of different native O157:H7 strains with an *rpoS*-containing vector appeared to increase adherence capabilities at different levels. In contrast, a decrease in adherence was observed in an O26:H11 strain upon complementation, while little difference was observed in the adherence abilities of an O111:H11 strain regardless of complementation with *rpoS*. The authors suggested that these differences in adherence could be attributed to differences in *rpoS* regulation of adherence mechanisms among serotypes. Alternatively, other sigma factors and/or transcriptional regulators maybe involved in these adherence mechanisms in the absence of RpoS. While studies such as these typically utilize laboratory test tube-grown cultures, there is little information known regarding the role of RpoS in the mediation of bacterial adherence in response to mechanical stresses such as LSMMG. *Salmonella* virulence potential has been shown to be dependent on RpoS regulation when grown under standard laboratory conditions (48). However, previous studies by Wilson *et al.* have shown that both wild-type and *rpoS* mutant strains of *S.* Typhimurium demonstrate an increased ability to tolerate different physiological stresses and survive in macrophages after growth under LSMMG (147). The authors suggest that the LSMMG environment can serve to pre-adapt *S.* Typhimurium to resist multiple stress environments, encountered during the course of infection, in an RpoS-independent manner. The findings presented here show, for the first time that the absence of RpoS expression can result in a hyper-adherent phenotype in an AIEC O83:H1 serotype grown under the conditions of LSMMG. To further understand the correlation between the absence of RpoS activity and the enhanced AIEC

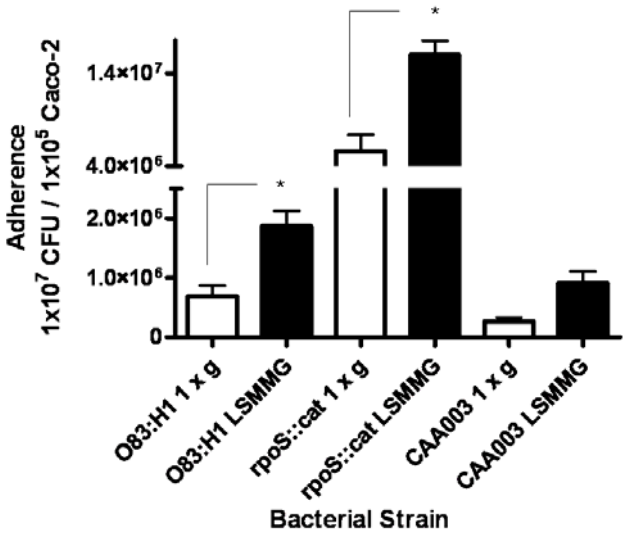
adherence phenotype in response to LSMMG, we next investigated the presence of RpoS-regulated adhesins/adhesion-associated factors which participate in the phenotype observed under LSMMG conditions.

Characterization of Tn*phoA* isolate CAA003 exhibiting reduced adherence phenotype under LSMMG conditions.

The elevated adherence demonstrated by *rpoS* mutant strain CAA001 lead us to investigate novel proteins contributing to this phenotype. To achieve this, we carried out transposon mutagenesis on the *rpoS* mutant strain to identify genes encoding putative adhesins and/or adhesion-associated proteins. We performed Tn*phoA* mutagenesis and identified 26 transposon mutants out of 10,000 colonies which were identified as dark blue colonies on media containing the substrate XP (5-bromo-4-chloro-3-indolyl phosphate). To look for mutants whose expression is mediated by RpoS, the 26 mutants were complemented with the plasmid pRpoS (generated from pGEMTEasy, Table 1), containing an intact copy of the *rpoS* gene, and screened again to look for the reversion of blue colonies back to colorless colonies. This screening process yielded two mutant strains which were further evaluated for changes in adhesion. A significant decrease in adherence was observed in one of the two isolates, which was named CAA003 (CAA001::Tn*phoA* isolate 1) (Fig. 10A). Strain CAA003 displayed reduced adherence in comparison to wild-type AIEC O83:H1 and CAA001 strains after growth under LSMMG (Fig. 10A). The decreased adherence suggested that the transposon had disrupted a gene encoding a putative adhesion-associated protein in CAA003. While the invasive ability of CAA003 was found to be comparable to that of the *rpoS* mutant under LSMMG conditions (Fig. 10B), overall invasion by the two strains was lower than that of the wild-type strain.

To further characterize the diminished adherence phenotype exhibited by CAA003, identification of the Tn*phoA*-disrupted gene within the genome of CAA003 was pursued. For this purpose, a cosmid library was generated and cosmids ligated with fragments containing the transposon were isolated and sequenced. BLAST results

A.



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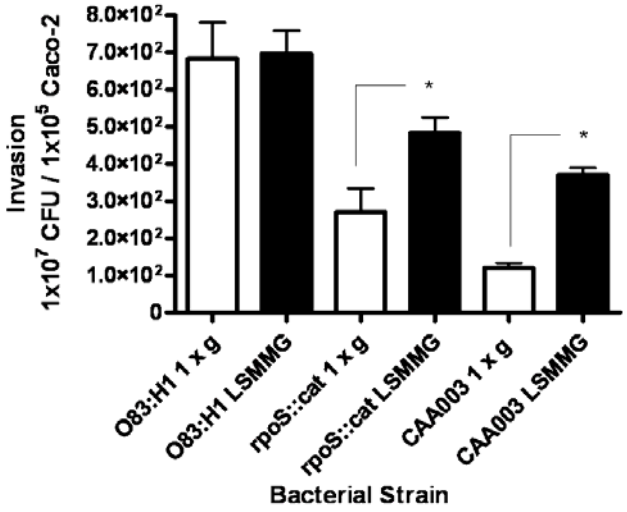


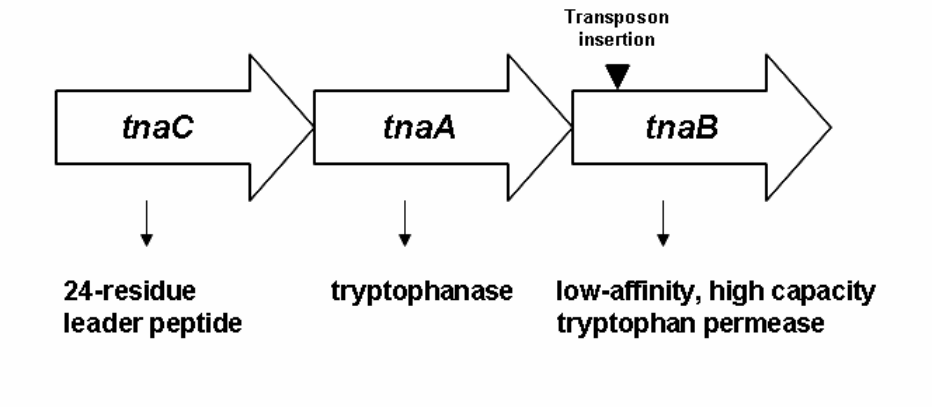
Figure 10. Adherence and invasion of CAA003 to Caco-2 cells. CAA003 (*rpoS*$^{-}$ *tnaB*$^{-}$) was grown under 1 x *g* control (□) or LSMMG (■) conditions then examined for changes in the ability to (A) adhere and (B) invade Caco-2 monolayers. (A) Both adhesion and invasion by LSMMG-grown CAA003 was found to be decreased in comparison to that of O83:H1 and CAA001. Data are representative of 2 independent trials. Asterisks indicate statistical significance ($p \leq 0.05$).

revealed that Tn*phoA* was inserted into the *tnaB* gene, which encodes a permease protein involved in tryptophan uptake. This gene is located in the *tnaCAB* operon, which is involved in tryptophan metabolism (Fig. 11A.). Next, complementation of CAA003 was carried out with an intact *tnaB* gene to determine if adherence capabilities could be restored to levels comparable to CAA001 or the wild-type strain. The vector pMD1 (kindly provided by C. Yanofsky, Stanford University), which contains an intact *tnaCAB* operon, was used to complement CAA003. Complementation restored adherence in CAA003 to levels higher than the wild-type strain and slightly lower than CAA001 (Fig. 11B).

These results suggest that the presence and expression of *tnaB* contributes to AIEC O83:H1 adherence activity under LSMMG conditions. Since the disrupted gene identified in CAA003 encodes a tryptophan-specific permease and not a known adhesin or adhesion-associated protein, the question remains how the *tnaCAB* operon and tryptophan metabolism influences adherence capabilities of AIEC O83:H1 under LSMMG conditions. Interestingly, expression by members of this operon have previously been reported to be essential in the processes of adherence, biofilm formation, and virulence in *E. coli*. Di Martino *et al.* have shown that a transposon insertion disrupting the *tnaA* gene, encoding tryptophanase, in *E. coli* strain S17-1 resulted in decreased adherence to A549 respiratory epithelial monolayers and in the ability to form biofilms on polystyrene (37). Complementation of the *tnaA* gene restored both adherence and biofilm capabilities. Anyanful *et al.* reported that generation of an isogenic *tnaA* mutant strain in the EPEC strain E2348/69 resulted with a loss of virulence in a *Caenorhabditis elegans* model (5). Tryptophanase activity was found to be necessary for full activation of the LEE1 promoter and in efficient formation of attaching and effacing lesions. Complementation of the mutant strain resulted in the restoration of the virulence phenotype.

While these reports showed that a number of effects were due to disruptions in the tryptophanase gene *tnaA*, tryptophanase activity was assessed to determine whether changes in activity had an impact on experimental results. Tryptophanase activity assays were carried out to determine if enzymatic activity by the operon in CAA001 and

A.



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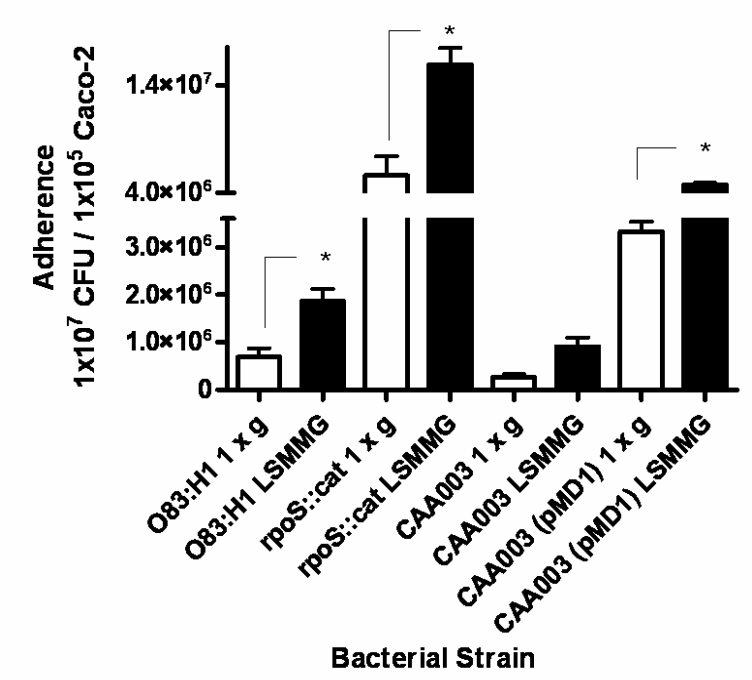

Fig. 11. Complementation of strain CAA003 with the *tnaCAB* operon restores adherence under LSMMG. (A) The tryptophanase (*tnaCAB*) operon. The three genes making up the operon include *tnaC* which encodes for a leader peptide sequence involved with Rho-dependent antitermination, *tnaA* which encodes for the enzyme tryptophanase which breaks down tryptophan, and *tnaB* which encodes a tryptophan-specific permease (the transposon insertion is indicated by [▼]). The CAA003 isolate was complemented with vector pMD1 (containing an intact *tnaCAB* operon) and grown under 1 x g control (□) or LSMMG (■) conditions to determine if adherence to Caco-2 monolayers is restored. Data are representative of 2 independent trials. Asterisks indicate statistical significance ($p \leq 0.05$).

CAA003 was altered in comparison to the wild-type strain. The assay involved the use of *S*-*o*-nitrophenyl-L-cysteine as a chromogenic substrate which can be degraded in the presence of tryptophanase forming *o*-nitrothiophenolate which can be measured photometrically (Fig. 12A). Tryptophanase-specific activity levels were found to be intact in both the CAA001 and CAA003 strains at levels exceeding the wild-type strain (Fig. 12B), indicating that disruption of the *tnaB* gene was the main variable impacting adherence activity. The elevated activity demonstrated by the *rpoS* mutant strains could be attributed to the absence of RpoS expression which could result in unregulated and enhanced tryptophanase activity by the operon. Lacour *et al.* has previously shown that RpoS plays a regulatory role in mediating the expression of genes in the *tnaCAB* operon and subsequent tryptophanase expression (76).

While there is evidence that members of the tryptophanase operon can alter adherence properties, there remains the question of what is the mechanism by which these effects are transduced. There is an increasing amount of evidence which indicates that indole, one of the by-products of tryptophan metabolism, can function as an external signaling molecule. Using *lacZ* fusions, previous studies have identified indole as the extracellular signaling factor in conditioned media from stationary phase *E. coli* cultures that was responsible for the activation of a number of different genes including *tnaB* (6, 145). The activity from the *tnaB*::*lacZ* fusion was found to be decreased greater than 70% after using conditioned media from a *tnaA* mutant that was unable to metabolize tryptophan and produce indole. In a subsequent study, Di Martino *et al*. demonstrated that exogenous supplementation of indole to an *E. coli* S17-1 *tnaA* mutant restored the ability of the mutant to form biofilms on polystyrene (36). The authors suggested that indole may act as a signaling molecule involved in mediating the expression of adhesion and biofilm-promoting factors.

These findings along with the experimental results presented in this study show that *tnaB* and other members of the *tnaCAB* operon play an important role in adherence and colonization processes in *E. coli*. Under LSMMG conditions, the absence of *tnaB* expression results with a decline in adherence capabilities by AIEC O83:H1 and complementation restores this adherence phenotype. One possible explanation for this

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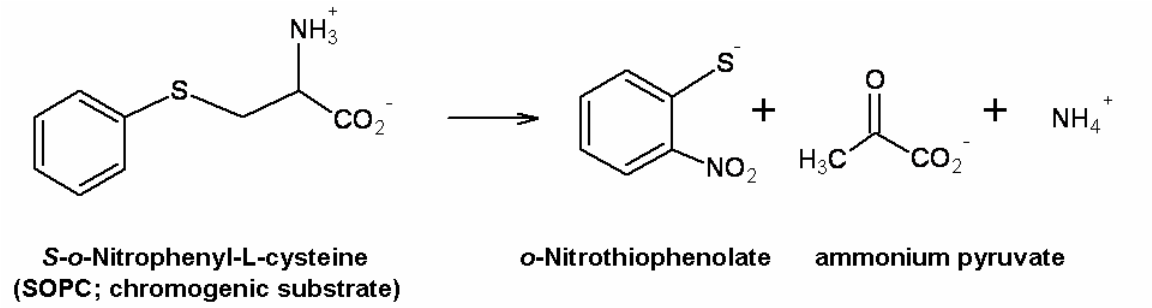

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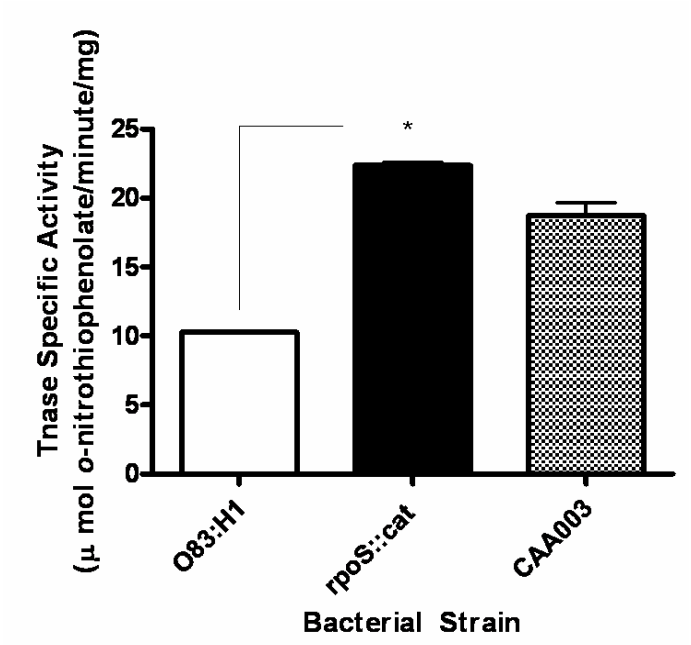

Figure 12. Tryptophanase enzymatic activity in AIEC O83:H1, *rpoS* mutant CAA001, and *rpoS*, *tnaB* mutant CAA003. (A) Enzymatic reaction by tryptophanase on *S*-*o*-nitrophenyl-L-cysteine (SOPC) chromogenic substrate producing *o*-nitrothiophenolate and ammonium pyruvate. (B) Tryptophanase-specific activity of AIEC O83:H1, *rpoS* mutant CAA001, and *rpoS*$^-$ *tnaB*$^-$ mutant CAA003. Data are representative of 3 independent trials. Error bars correspond to standard error of means generated from triplicate cultures. Asterisks indicate statistical significance ($p \leq 0.05$).

trend is that the loss of *tnaB* expression prevents normal expression of the tryptophan permease responsible for tryptophan uptake and import into the cell. A decrease in the amount of intracellular tryptophan directly reduces the production of indole through tryptophan metabolism, which can affect downstream signaling processes dependent on the activation by this extracellular signaling molecule. These processes could include the expression of genes encoding proteins which may play a role in adherence-based processes.

CONCLUSIONS

The research findings presented in this dissertation show how mechanical stresses, such as microgravity and low-shear stress, can alter the genomic profile of Gram-positive bacteria (e.g. *S. pneumoniae*) as well as the physiology and colonization properties of adherent-invasive *E. coli* isolates associated with Crohn's Disease. The overall trend of down-regulated genes observed by *S. pneumoniae* in response to LSMMG conditions included genes physically located throughout the genome, which represented multiple functional categories and included genes of common operons / regulons. While previous work by Niesel *et al.* (unpublished data) has demonstrated an increase in virulence potential by *S. pneumoniae* after growth under LSMMG, the induction of known virulence genes was not observed to explain these findings. The genomic responses to the LSMMG environment could suggest that the lack of virulence gene expression might be contributing to immunoevasive strategies elicited by the bacteria to avoid detection by the host immune system. Alternatively, LSMMG-mediated responses could be promoting the induction of novel genes and mechanisms associated with virulence function and / or the fine-tuning of characterized genes and mechanisms in a different way to promote virulence-associated functions. Future investigations can be pursued to correlate the gene expression trends presented here with changes at the protein level. Determining the putative roles for such proteins in survival and virulence-associated processes will help substantiate the importance of LSMMG-mediated signaling and its influence on transcriptional regulation and subsequent protein expression. In addition, the potential roles of LSMMG-responsive genes at the cellular level can be determined through mutagenesis-based studies involving physiological comparisons (e.g. stress resistance and virulence potential) between wild-type and mutant strains in response to the LSMMG environment.

Physiological analyses of AIEC strain O83:H1 after growth under LSMMG revealed both alterations in environmental stress resistance and colonization potential. Alterations in stress resistance (e.g. increased stress resistance and sensitivity) appeared to be both LSMMG and *rpoS*-dependent in a stress-specific manner. The absence of

rpoS expression under LSMMG conditions conferred a hyper-adherent phenotype which involved participation of the tryptophan permease, encoded by the gene *tnaB*. Though no genes were identified which encode known adhesins or adhesion-associated proteins, a possible mechanism through which the *tnaCAB* operon could be contributing to adherence is by the production of the tryptophan metabolite indole. This compound has previously been shown to function as an extracellular signaling molecule associated with the restoration of adhesion and biofilm formation capabilities in mutant strains (89, 145). To better understand to potential contribution of indole in adherence function, future studies should address changes in the global gene profiles of LSMMG-grown cultures with and without indole supplementation. In addition, further analyses addressing RpoS regulation in AIEC at the transcriptional, translational, and post-translational levels could provide information which can be used to develop a more comprehensive understanding of the molecular mechanism(s) and key mediators used by bacteria to respond and adapt to mechanical forces such as LSMMG.

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Vita

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Publications

(Peer-Reviewed Articles)

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