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Lee W. Goneau, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Microbiology and Immunology

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SUB-INHIBITORY ANTIBIOTICS ENHANCE VIRULENCE, PERSISTENCE, AND PATHOGENESIS OF UROPATHOGENS

(Thesis format: Monograph)

by

Lee William Goneau

Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

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Abstract

In addition to their bactericidal effects, antibiotics are potent signal mediators at sub-inhibitory levels in the environment. The ability to modulate community structure in this niche raises concerns over their capacity to influence pathogenesis in patients during antibiotic therapy. This concept forms the basis of this thesis, and is explored using models of prophylactic therapy for recurrent urinary tract infection (UTI) management.

Sub-inhibitory ciprofloxacin, ampicillin, and gentamicin were found to augment virulence *in vitro*, increasing adherence and urothelial cell invasion in uropathogenic *Escherichia coli* (UPEC) and *Staphylococcus saprophyticus*. In addition, biofilm formation was increased, and swarming motility decreased. In UPEC, the effect of antibiotics on these processes was abolished in SOS-deficient strains.

Trans-urethral inoculation of mice with ciprofloxacin-primed *S. saprophyticus* or UPEC significantly increased bacterial burden in both bladders and kidneys at one and 14 days post-inoculation (dpi). Sub-therapeutic ciprofloxacin supplemented in the drinking water of chronically infected mice significantly increased bacterial urine load. In addition, mice previously infected but clinically resolved suffered recurrences. These mice had impaired urinary polymorphonuclear leukocyte infiltrates, in part due to antibiotic-dependent cytokine suppression during initial infection. Prophylactic intervention had no significant effect on UPEC clearance, but did significantly increase bacterial intracellular bladder reservoirs, raising concerns over the clinical efficacy of this management strategy and risks of promoting persistent infection.

The inability of antibiotics to clear infection in prophylaxis models was attributed to the presence of MDT persister cells. Sub-inhibitory antibiotic pre-treatments were found to increase persister fractions, but this effect was abolished in SOS-deficient strains. Conducting these assays with UPEC isolated from recurrent UTI patients revealed an enriched persister fraction compared to organisms cleared with standard antibiotic therapy, suggesting persister traits are either selected for during prolonged antibiotic treatment or initially contribute to therapy failure.

This work represents the first attempt to illustrate that observed sub-inhibitory, pathogen associated antibiotic-dependent changes *in vitro* have significant *in vivo* consequences. It is hoped that this research will lead to a re-examination of how antibiotics are administered for management of patients suffering from recurrent UTI and other chronic diseases.

Keywords

Staphylococcus saprophyticus, *Escherichia coli*, UPEC, MDT, recurrent UTI, antibiotic, urothelium, murine model, uropathogenesis, hormesis, urothelial immunity.

Co-Authorship Statement

The work herein contains material from previously published manuscripts as well as manuscripts that are in preparation. It is based upon portions of the following three manuscripts:

Sections 3.1.2 (Evaluating the ability of sub-inhibitory antibiotics to induce adherence of *S. saprophyticus* to abiotic surfaces), 3.1.5 (Antibiotics induce bacterial aggregation), and 3.2.6 (Evaluating the capacity of ciprofloxacin to modulate urothelial immune responses) are based upon a publication in the *Journal of Endourology* (Erdeljan P., MacDonald, K. W., Goneau, L. W., Bevan, T., Carriveau, R., Razvi, H., Denstedt J. D., and Cadieux, P. A. 2012. Effects of subinhibitory concentrations of ciprofloxacin on *Staphylococcus saprophyticus* adherence and virulence in urinary tract infections. *J Endourol*. **26**, 32-37). My contributions were in study and experimental design, slide and stent adherence assays, SEM, and in the writing of the manuscript.

Section 3.3 (Understanding the ability of uropathogens to persist despite adequate antibiotic intervention during infection) is based upon a publication in *Antimicrobial Agents and Chemotherapy* (Goneau, L. W., Yeoh, N. S., MacDonald, K. W., Cadieux, P. A., Burton, J. P., Razvi, H., and Reid, G. 2014. Selective target inactivation rather than global metabolic dormancy causes antibiotic tolerance in uropathogens. *Antimicrob Agents Chemother.* AAC-02552). Nigel Yeoh conducted SSR and AI persister characterization experiments. Kyle MacDonald was involved in experimental design and the writing of the manuscript. Drs. Cadieux, Burton, and Reid were involved in study design and critical review of the manuscript. Dr. Hassan Razvi provided clinical expertise in the area of recurrent UTI and was involved in critically reviewing the manuscript.

Significant portions of Sections 3.1 (Examining the effects of sub-inhibitory antibiotics on uropathogen virulence) and 3.2 (Demonstrating the influence of sub-inhibitory ciprofloxacin on uropathogenesis in a murine model of UTI) are based upon a manuscript currently in preparation (Goneau, L. W., Hannan, T. J., Gloor, G. B., MacPhee, R. A., Scwartz, D. J., Razvi, H., Burton, J. P., Hultgren, S. J., and Reid, G.).

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List of Abbreviations

ADEP4	acyldepsipeptide
ADP	adenosine diphosphate
AI	acute infection
AL	Alabama
ALDEx	ANOVA-like differential expression
AMP	ampicillin
ANOVA	analysis of variance
Arr	aminoglycoside response regulator
ATP	adenosine triphosphate
AUA	American Urological Association
AUM	asymmetrical unit membrane
BCA	bicinchoninic acid
BLAST	basic local alignment search tool
bp	base pair
BSL	biosafety level
cAMP	cyclic adenosine monophosphate
c-di-GMP	cyclic diguanylate
cfu	colony forming unit
CIP	ciprofloxacin
CLSI	clinical laboratory standards institute
CXCR1	cysteine X cysteine chemokine receptor
°C	degree celsius
dH_2O	deionised water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
dpi	days post-inoculation
EC	*E. coli*
eDNA	extracellular DNA
EDTA	ethylenediaminetetraacetic acid
EDX	energy dispersive X-ray

EHEC	enterohemorrhagic *E. coli*
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential medium
FBS	fetal bovine serum
FDA	food and drug administration
FOV	fields of view
GA	Georgia
GAS	group A streptococci
G-CSF	granulocyte colony-stimulating factor
GEN	gentamicin
GFP	green fluorescent protein
HGT	horizontal gene transfer
hpf	high-powered field
hpi	hours post-inoculation
HPU	human pooled urine
HUS	hemolytic-uremic syndrome
IBC	intracellular bacterial community
IL	interleukin
IN	Indiana
IPTG	isopropyl β-D-1-thiogalactopyranoside
IS	insertion sequence
KAAS	KEGG Automatic Annotation Server
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG orthology
KC	keratinocyte-derived chemokine
LB	lysogeny broth
LPS	lipopolysaccharide
MA	Massachusetts
MDR	multidrug resistant
MDT	multidrug tolerant
MH	Mueller Hinton
MIC	minimal inhibitory concentration

MN	Minnesota
MO	Missouri
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MRSA	methicillin resistant S. aureus
NBCI	National Center for Biotechnology Information
nt	nucleotide
NY	New York
OD	optical density
OH	Ohio
ON	Ontario
ORF	open reading frame
PA	Pennsylvania
(p)ppGpp	guanosine pentaphosphate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	power of hydrogen
PIA	polysaccharide intercellular adhesin
PKA	protein kinase A
PMN	polymorphonuclear leukocyte
PMSF	phenylmethylsulfonyl fluoride
PSM	phenol-soluble modulin
PVL	Panton-Valentine leukocidin
QIR	quiescent intracellular reservoir
RBC	red blood cell
RIN	RNA integrity number
RNA	ribonucleic acid
rpm	rotations per minute
SEM	scanning electron microscope
Spe	streptoccocal pyogenic exotoxin
spp.	species
SS	*S. saprophyticus*

Ssp	surface-associated lipase
SSR	same-strain recurrence
Stx	shiga-toxin
TA	toxin/antitoxin
TAE	tris acetate EDTA
TBE	tris borate EDTA
TEM	transmission electron microscope
THP	Tamm-Horsfall protein
TLR	Toll-like receptor
TMP/SMX	trimethoprim-sulfamethoxazole
TNFα	tumor necrosis factor alpha
Uaf	uro-adherence factor
UPEC	uropathogenic *E. coli*
US	United States
UT	Utah
UTI	urinary tract infection
UV	ultraviolet
VT	Vermont
VUR	vesicoureteral reflux
WGA	wheat germ agglutinin
WT	wild-type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZOI	zone of inhibition

CHAPTER ONE

INTRODUCTION

1 INTRODUCTION

1.1 THE INFLUENCE OF ANTIBIOTICS ON HOST AND PATHOGENS

Since their discovery, antibiotics have been used with overwhelming success in treating and preventing infections, saving countless lives along the way. However, in recent times, questions have been raised about not only adverse effects and multi-drug resistance, but also the natural role of antibiotics in the environment and the potential role in complicating disease (Yim *et al.*, 2007; Romero *et al.*, 2011) and affecting long term health (Blaser, 2011). Thus, a potential paradigm shift is emerging on how we view antibiotics. A number of case reports and clinical trials corroborate this shift, with a growing regimen of antimicrobials associated with increased morbidity and mortality when used for purposes outside their recommended guidelines (Harbarth *et al*., 2003). It is critical that inappropriate prescription practices cease and that the current issues associated with antibiotic misuse be brought to light.

1.1.1 A wider perspective of how antibiotics affect microorganisms

Following Alexander Fleming's discovery that microorganisms secrete antimicrobial agents (Fleming, 1929), there has been an explosion in the number of compounds and synthetic derivatives that have become available for therapeutic use. Unsurprisingly, soil organisms such as *Streptomyces* spp. and the penicillin-producing *Penicillium notatum*, first observed by Fleming have been the indispensible work-horses of the chemotherapeutic field, supplying the vast majority of antimicrobial compounds used clinically today. However, it is surprising, perhaps even unnerving, that the natural roles of these small molecules in the environment from which they are derived are still largely unknown, with only a fraction of natural therapeutic products having been extensively studied (Romero *et al.*, 2011). It had long been thought that antibiotics functioned simply as molecular weapons, whose inhibitory properties we readily take advantage of

clinically. We now know that they have diverse effects on microorganisms, depending on the dose administered (Davies *et al*., 2006). Many of these compounds are stimulatory at doses of less than the minimal inhibitory concentration (MIC) and are capable of modulating the expression of upwards of 5-10% of the entire bacterial genome (Goh *et al*., 2002) in a bimodal dose-response relationship referred to as hormesis (Southam and Erlich, 1943). A growing body of literature now supports their role as interspecies signalling molecules involved in optimizing multi-organism community structure and function (Bernier *et al.*, 2013).

Antibiotics can meet many of the criteria for functioning as signalling agents, whose production occurs during tightly regulated stages of growth, under certain physiological conditions, with accumulation generating a concerted response once a certain population threshold is achieved (Keller and Surette, 2006; Winzer *et al*., 2002; Hibbing *et al.*, 2010). The utility of this tight regulation is that antibiotics are delivered by an entire population at a very specific concentration, often which is many fold-lower than what is prescribed therapeutically and insufficient for antibiosis (Yim *et al.*, 2007). Recent investigation of natural populations of *Streptomyces* spp. demonstrated that sub-inhibitory antibiotics are important regulators of interspecies nutrient utilization strategies, and are effective in reducing niche overlap in mixed populations (Jauri *et al.*, 2013). In a seminal paper, Hoffman *et al.* (2005) demonstrated that sub-inhibitory aminoglycosides were capable of inducing bacterial biofilm formation in *Pseudomonas aeruginosa*. This response was mediated by the aminoglycoside response regulator (*arr*) gene, whose activity modulated levels of the major regulator c-di-GMP, influencing several genes important to motility and pili production amongst others. This interaction with a specific regulator supports the claim that antibiotics can have precise signalling targets.

Other agents have similar effects but also up-regulate expression of secretion system genes. This increases cytotoxicity when challenged against a macrophage cell line and increases competition against other organisms in the environment (Linares *et al.*, 2006; Jones *et al.*, 2013). Antibiotics can influence the larger microbial community structure by hijacking inter-species regulatory systems of niche inhabitants. In *Streptococcus pneumoniae*, sub-inhibitory penicillin stimulates the production of the inter-bacterial

signalling agent autoinducer-2 (AI-2), a factor important in biofilm formation in many species (Rogers *et al.*, 2007). The culmination of these responses in the environment may be to repel or defend against predatory eukaryote attack, which may be stimulated by 'sensing' streptomyces, providing an obvious benefit to multispecies community lifestyles. Clearly, such antibiotic-induced changes may also have significant ramifications for patients receiving antibiotic therapy. This new understanding of antibiotic functions in natural environments necessitates a re-evaluation of their use in the clinical setting.

1.1.2 Sub-inhibitory antibiotics are capable of modulating bacterial virulence and pathogenesis

Clinicians are well aware of the common side-effects of antibiotic therapy which may include microbiota dysbiosis (gastro-intestinal distress, oral and vaginal candidiasis, bacterial vaginosis, skin rashes), the establishment of resistant variants and host toxicity (Dancer *et al.*, 2004). Worsening patient prognosis is often attributed to confounding factors such as patient immune status, while the actual cause may be the prescribed agent itself. Here, the effects that antibiotics have on bacterial virulence will be explored, citing patient trials when possible, but relying mostly on the growing body of *in vitro* data.

Effects on modulation of toxin production

Since Julian Davies' group began the first serious investigations of sub-inhibitory antibiotic hormetic effects using promoter-luciferase expression libraries over a decade ago (Goh *et al.*, 2002), there has been an increasing focus on characterizing phenotypes relevant to pathogenesis. The changes that sub-inhibitory antibiotics invoke can be dramatic and highly variable depending on the class and the organism challenged. Even slight modifications of chemical groups within the same antibiotic can affect its ability to augment pathogenesis (Nielsen *et al.*, 2012). However, some antibiotic classes are associated with specific responses. For example, protein synthesis inhibitors generally suppress toxin expression while DNA and cell wall acting agents enhance it. These differences are likely attributed to their mechanism of action, with protein synthesis

inhibitors preventing toxin mRNA translation and subsequent release when supplied at appropriate levels. There may be several routes by which antibiotics modulate gene expression, involving specific signalling and general stress response systems (Hoffman *et al.*, 2005; Kelley, 2006). Knowledge of how these responses augment pathogenesis will aid physicians in disease management for various infection milieu.

The most characterized case of antibiotics directly influencing bacterial virulence is through the upregulation of shiga-toxin (Stx) production in enterohemorrhagic *Escherichia coli* (EHEC) and induced onset of hemolytic-uremic syndrome (HUS). This medical emergency carries a 5-10% mortality rate, and inappropriate antibiotic application is significantly associated with increased HUS risk from ~15 to ~50% (Wong *et al.*, 2000; Serna IV and Boedeker, 2008). Antibiotic stress can drive bacteriophages from lysogeny, resulting in phage replication during the lytic cycle and concurrent production of phage related genes such as Stx. In the EHEC epidemic strain O157:H7, sub-inhibitory fluoroquinolone and co-trimoxazole drive Stx production, while antibiotics that target cell-wall, transcription and translation do not (McGannon *et al.*, 2010). Conversely, other agents are capable of inhibiting Stx production even at low levels (McGannon *et al.*, 2010). The reaction of organisms to antibiotics is highly variable, and is determined by the agent provided and targeted strain. Importantly, phage activation is not a requirement of antibiotic-induced virulence. Other mechanisms appear to exist which are much less well characterized but believed to be driven by changes in stress response-related systems.

Non-phage associated virulence traits can also be induced with antibiotics. In group A streptococci (GAS), clindamycin and benzylpenicillin are commonly used therapeutics for ameliorating toxic shock-like syndromes by suppressing exotoxin SpeA/B production (Mascini *et al*., 2001). However, these and protein synthesis-inhibitors also increase levels of other potent toxins including streptolysin and streptococcal inhibitor of complement at sub-inhibitory levels (Tanaka *et al.*, 2005). The effect of these agents is abrogated when supplied at levels higher than the MIC, highlighting the importance of maintaining therapeutic levels during treatment. This may be difficult when organisms are either highly tolerant or resistant, such as in methicillin resistant *Staphylococcus aureus* (MRSA). In these scenarios a combination of antibiotic ineffectiveness and

induced virulence expression could easily exacerbate an already complicated infection. The observation that resistant organisms are also in many cases hypervirulent may be a direct, yet unexplored consequence of inappropriate antibiotic therapy (Fluit, 2005). Indeed, there is a trend linking inappropriate therapy of resistant infections with negative outcomes in patients, and not just because of antibiotic failure (Dancer *et al.*, 2004). Dumitrescu *et al.* (2011) demonstrated the induction of Panton-Valentine leukocidin (PVL) toxin in MRSA when various antibiotics interfered with penicillin binding proteins. Conversely, clindamycin and linezolid inhibit the release of PVL and other toxins (Dumitrescu *et al.*, 2007; Otto *et al.*, 2013). Some protein synthesis inhibitors increase expression of the *agr* virulence gene regulator in MRSA (Joo *et al.*, 2010), resulting in the production of phenol-soluble modulins (PSM) responsible for neutrophil lysis and pro-inflammatory responses. Toxin production is likely attributable to sub-inhibitory dosing which induces gene expression but is inadequate to prevent ribosomal translation (Joo *et al.*, 2010). This could be clinically relevant during infections caused by resistant organisms which have a greater threshold for tolerating therapeutic dosing. Because there is a strong epidemiological link between PVL/PSM and severe infections such as necrotizing pneumonia, recurrent complicated osteomyelitis and sepsis (Mitchell *et al.*, 2007), empirical use of certain antibiotics in locations with high MRSA incidence should be avoided.

Effects on bacterial adherence, invasion and biofilm formation

In resistant and non-resistant organisms, many antibiotics induce a hyper-adhesive phenotype that is characterized by an increase in surface adhesin production (Rasigade *et al.*, 2011; Bisogano *et al.*, 2000; Denève *et al.*, 2009; Erdeljan *et al.*, 2012). The increased capacity to adhere to tissues and medical device surfaces are of particular concern as it potentiates the formation of antibiotic tolerant biofilms (Hoffman *et al.*, 2005). Once a biofilm is established, MIC shifting occurs, which may permit increased toxin production in the now tolerant organisms. In addition, sub-inhibitory antibiotics can promote the rapid formation of planktonic *S. aureus* aggregates with lowered susceptibility to antibiotic killing (10-100x less susceptible) (Haaber *et al.*, 2012; McNabe *et al.*, 2011).

These biofilm-like communities may be less susceptible to phagocytic immune predation (Foster, 2005), providing multivariate benefits to survival and persistence within the host. Kaplan *et al.*, (2012) demonstrated that resistant isolates had higher fold-change in biofilm formation compared to sensitive strains following antibiotic exposure. In biofilm-associated pathogens, such as *Staphylococcus* spp., antibiotic-induced biofilm formation likely occurs in both *icaADBC*-dependent (polysaccharide intercellular adhesin - PIA) and independent (proteinaceous and eDNA) manners (Wang *et al.* 2010; Rachid *et al.*, 2000), involving activation of both specific sensors (Hoffman *et al.*, 2005) and general stress responses (Li *et al.*, 2005[a]). Additionally, increased adherence also potentiates hyperinvasive phenotypes in some organisms including multidrug resistant (MDR) *Salmonella enterica* serovar Typhimurium (Brunelle *et al.*, 2013). In this case, antibiotics cause the circumvention of temporally-regulated gene expression, inducing early and fully invasive phenotypes by upregulating hilA, prgH and invF. Similar to several other cases, this phenotype was found not to be ubiquitous, and did not occur in all *S. enterica* strains. Increasing the ability of organisms to adhere, internalize, and form antibiotic tolerant biofilms subverts early immune responses and increases infection severity. Of note, the vast majority of studies conducted thus far have relied on *in vitro* models, with *in vivo* characterization not considered. Clearly, the latter types of investigation are required to ensure the clinical relevancy of these observations, and to raise concern over the legitimacy of these claims. This formed a component of the current thesis.

1.1.3 Immunomodulatory effects of antibiotics

In addition to their potent bactericidal properties, some antibiotics have been demonstrated to have immunomodulatory functions which can affect the host. The majority of these responses seem to limit immune activation, and have been demonstrated primarily in macrolide and quinolone agents (Kanoh and Rubin, 2010; Dalhoff and Shalit, 2003). Most antibiotics which demonstrate immunomodulatory properties appear to function by augmenting the release of pro-inflammatory cytokines. Specifically, macrolides are well documented in dampen the release of interleukin (IL)-8 and tumor necrosis factor alpha (TNFα) (Čulić *et al.*, 2001), while fluoroquinolones are capable of

suppressing IL-1α and TNFα, and superinducing IL-2 (Araujo *et al.*, 2002; Riesbeck *et al.*, 1998; Anderson *et al.*, 2010). Although it is largely unknown how antibiotics modulate these processes, interruption of normal protein kinase A (PKA) activity is believed to play a role (Song *et al.*, 2007). Antibiotics exhibiting immunomodulation often demonstrate phosphodiesterase inhibition which results in cellular cAMP accumulation, superoxide anion generation and subsequent inhibition of cytokine production (Blaine *et al.*, 1997; Ono *et al.*, 2000). Macrolides have been shown to reduce the stability and translation of cytokine transcripts resulting in decreased production (Wax *et al.*, 2003). This in turn results in decreased immune cell infiltrate to the sites of infection or damage. It appears as though antibiotics do not suppress the immune response *per se*, but rather normalize it to basal activation levels, effectively dampening otherwise normal responses following stimulation (Shinkai *et al.*, 2008).

The modulatory properties of several antibiotics have already been taken advantage of clinically. For example, macrolide application has demonstrated improved outcome in patients suffering with chronic inflammatory pulmonary diseases (Cameron *et al.*, 2012). These agents were also associated with decreased in-hospital mortality during treatment of community-acquired pneumonia compared to other antibiotics (Martinez, 2004). Direct effects on autoimmune diseases such as rheumatoid arthritis have been demonstrated, with minocycline decreasing disease activity and tender joint count (Stone *et al.*, 2003). Together, these studies suggest that immune-related influences can be capitalized upon to yield positive outcomes for many diseases. However, immunosuppression also comes at a price, introducing a greater risk of patients going on to develop more severe infections including chronic disease, bacteremia, and potentially fatal sepsis (Gea-Banacloche *et al.*, 2004). Therefore, characterization of these agents in different disease milieu is certainly warranted.

1.1.4 Clinical implications of inadequate antibiotic therapy

When antibiotics are prescribed, there are many pharmacokinetic, pharmacodynamic, host, and bacterial properties which can keep them from either reaching their full therapeutic potential, or decrease the period that they remain at levels greater than the

MIC (Ambrose *et al.*, 2007). These transient non-suppressive periods provide pathogens with a window to replicate, evolve resistance and potentially express toxins. Therefore, outlining and understanding factors which limit these periods are critical for improving efficacy. A major problem with antibiotic administration today is that many management strategies are based on expert opinions and not empirical evidence indicating positive outcomes. For example, long-term, prophylactic approaches have not been altered in decades despite questionable efficacy and long-term effectiveness (Mattoo *et al.*, 2009), and most of the 'recommended' drugs have not been formally approved by the US FDA for prophylaxis (Enzler *et al.*, 2011). This could lead to inappropriate or unnecessary administration of antibiotics for problems that could have been resolved by different approaches. Therefore, it is critical for clinicians to understand how the antibiotics that they prescribe concentrate in the body and identify and address factors which might influence this process.

The concentration of antibiotics at therapeutic sites is not always determined during drug development, with plasma concentration regarded as an appropriate marker for the provision of efficacious dose, despite reportedly high interpersonal variation (Drusano, 2004). Recent studies have revealed poor correlation between vascular concentrations and those within the soft-tissue of patients for some antibiotics, with levels often never reaching therapeutic potential in certain sites (Matzneller *et al.*, 2013). The situation is worse in patients undergoing prophylactic therapy, where antibiotics are present at low levels for extended periods (Pomeranz *et al.*, 2000). Single dose therapies are at particular risk of falling below the organism's MIC threshold (Dvorchik *et al.*, 2003). It is surprising that many prophylactic therapies are still used in practice despite their questionable effectiveness and demonstrated potential to increase the risk of developing resistant infections (Conway *et al.*, 2007; Mattoo *et al.*, 2009). Counter-intuitively, some preventative prophylactic strategies aimed at reducing hospital-acquired infections have resulted in more severe and numerous complications (Madden *et al.*, 2010). Similarly, perioperative systemic prophylaxis does not necessarily reach uniformly therapeutic levels at all anatomical districts and may introduce the potential for off-target effects (Campoccia *et al.*, 2010). Physician compliance in following dosing guidelines is reportedly poor, with many failing to cease therapy after surgery (Namias *et al.*, 1999), or

disregarding intraoperative re-dosing when recommended (Miliani *et al.*, 2009). Inadequate intraoperative or excessive post-surgery dosing is associated with increased patient risk of bacteremia, and line and surgical site infections (Namias *et al.*, 1999; Miliani *et al.* 2009). Although these authors conclude no causative relationship for increased infection risk following inadequate or inappropriate therapy, it is possible that the sub-inhibitory levels achieved *in situ* are modulating the virulence potential of would-be pathogens. This may explain why short courses of antibiotic therapy can increase the risk of developing secondary infections, for example sepsis following transrectal ultrasonography-guided biopsy (Patel *et al.* 2011). In addition, patients having received antibiotics within a month prior to sepsis onset are less responsive to therapy and associated with worse prognosis (Garnacho-Montero *et al.*, 2003). It is perhaps not surprising that the increasing incidence of sepsis over the last 40-50 years may be a result of increasing antibiotic use (Martin *et al.*, 2003; Roumie *et al.*, 2005; McCaig and Hughes, 1995).

Other overlooked factors which can influence the levels of antibiotics in the body include patient health (diabetes, ischemia, hepatic/renal dysfunction, sepsis), compliance and size (Patel *et al.*, 2010; Ambrose *et al.*, 2007). Diseases such as obesity which are becoming epidemic worldwide merit special consideration as physiological alterations can occur which affect distribution and clearance of antibiotics (Janson and Thursky, 2012). This suggests that the 'one-size-fits-all' dosing strategies are outdated, but can be improved upon by considering individual patient parameters to arrive at appropriate dosing regimens (Falagas and Karageorgopolos, 2010). Studies of the antibiotic-pathogen microenvironment indicate factors affecting bodily distribution need to be addressed, as the niche occupied by an organism influences its specific response to antibiotics (Zhang *et al.,* 2011; Frisch *et al.*, 2011). Recently, the development of antibiotic-leaching medical devices has been proposed to deliver agents at local wound sites while avoiding the unfavourable side-effects of systemic therapy. However, as is the case with many prophylactic therapies, there is an absence of strict harmonized guidelines for the use of these coated biomaterials (Campoccia *et al.,* 2010). Specifically, there is no guarantee that the desired delivery curve is achieved and that adequate antibiotics diffuse into the surgical site in the appropriate period of time. It is possible that organisms could tolerate

therapy in what has been referred to as persister states until the device elutes sub-inhibitory antibiotics and trigger a new infection. This seems to be the case for one patient, who was left with gentamicin eluting beads *in situ* for several years and went on to develop resistant infection at the implant site (Neut *et al.*, 2003). For these reasons, it has been suggested that the personal and financial ramifications of using antibiotic-impregnated devices may outweigh any associated benefits (Cummins *et al.*, 2010).

Together, these studies present overwhelming evidence that the types of antibiotics and dosages prescribed by primary caregivers can have broad ranging adverse side effects on patients. Inappropriate antibiotic prescriptions have resulted in the loss of life and prolonged hospitalization in the past and are unfortunate but avoidable consequences of non-specific guidelines and outdated policies (Dancer *et al.*, 2004; Spoorenberg *et al.*, 2014). Of particular concern is the proposed capacity of antibiotic to propagate chronic disease such as recurrent urinary tract infection (UTI) through prophylactic application, a practice which is still widely adopted today despite questionable efficacy. Indeed, anecdotal evidence has suggested that increased frequency of recurrent UTI correlates with widespread antibiotic use (Nickel, 2005). Therefore, the capacity of antibiotics to affect disease severity during recurrent UTI is considered in this work.

1.2 URINARY TRACT INFECTIONS

UTIs represent one of the most common diseases caused by bacteria. In 2006 alone, UTIs were the cause of 11 million physician visits, 1.7 million emergency room visits and cost the US health care system 3.5 billion dollars (Nielubowicz and Mobley, 2010). In addition to their high community incidence rate, at 40%, UTIs are also the most common cause of hospital acquired infection (Klevens *et al.*, 2002). The female lifetime incidence is roughly 50%, with 44% of those infected going on to suffer recurrences within one year of initial infection (Raz *et al.*, 2000). Infections in the elderly and prepubescent are also common, with subsequent potential for renal scarring in infants carrying severe consequences later in life. Comparatively, men suffer from UTI much less often, with most infection episodes related to anatomical abnormalities.

Risk factors for uncomplicated UTI in otherwise healthy individuals include intercourse frequency, spermicide use, and familial or personal history of UTI (Foxman, 2010). Lower tract involvement includes bladder (cystitis) and urethral infection, while upper tract infections involve the kidneys (pyelonephritis) and ureters. Sub-populations of individuals with underlying urological abnormalities, spinal cord injury, medical device implantation, and diabetes are further susceptible to UTI and classified as complicated cases (Foxman, 2010). Cystitis symptoms, which in part are due to mucosal immune responses, often include burning and painful micturition, cloudy and odorous urine, frequent voiding, urgency and abdominal discomfort, while pyelonephritis is also characterized by fever, nausea, vomiting, and flank pain. These symptoms are often accompanied by additional signs including gross hematuria (bloody urine) and pyuria (the presence of 6-10 polymorphonuclear leukocytes per high powered field of unspun, voided midstream urine).

Although UTI's can develop into potentially life threatening conditions (eg. pyelonephritis, sepsis, kidney failure), most are relatively benign and often self-resolve without the need for clinical intervention (Christiaens *et al.*, 2002; Reid *et al.*, 2010). Despite this, the sheer number of people affected [150 million UTIs per annum worldwide (Stamm and Norrby, 2001)], the economic burden from antibiotic prescriptions [one fifth of all oral antibiotics prescribed in Ontario are for treatment of

UTI (Daneman *et al.*, 2011)], and capacity to cause serious deterioration in the quality of life supports the need for continued research in this field.

1.2.1 Contribution of host immune function to infection risk

Host genetics strongly influence an individual's susceptibility of developing recurrent, chronic and severe UTI. For example, one study found that 42% of family members of patients with a history of UTI were UTI-prone compared to only 11% for controls who did not have a history of UTI (Stauffer *et al*., 2004). These hereditary links are believed to manifest in part due to the immune functionality of the host. Therefore, significant research focus is dedicated to flushing out genetic links which dictate UTI susceptibility, with recent studies yielding perplexing findings. Primary resistance to UTI is determined by the innate immune system, especially during acute phases of infection. Alterations in innate immune functionality can either exacerbate infection or impart protective benefits. For example, polymorphisms in genes which affect Toll-like receptor (TLR)-4 signalling are associated with decreased immune infiltration and resulting tissue damage. Despite their immunodeficiencies, rather than developing more severe infection these individuals are often associated with asymptomatic carrier states devoid of typical UTI pathology (Fischer *et al.*, 2006). In addition, a polymorphism which decreased TLR4 signalling *in vitro* reduced the risk of recurrent UTI in premenopausal women (Hawn *et al.*, 2009). Conversely, children prone to pyelonephritis typically demonstrate a reduced neutrophil infiltrate following *E. coli* infection, often due to deficiencies in the CXCR1 receptor (Svensson *et al.*, 2011). These patients are at high risk of going on to suffer from severe complications of infection including the development of urosepsis. This dichotomy reveals the sensitivity of disease outcome to initial stages of infection, and are likely dependent on the cytokine profiles which are generated in response to UTI. This has been confirmed in mouse studies which reveal early severe inflammatory responses to infection predispose to chronic and recurrent disease. In these mice, elevated IL-6, G-CSF, and IL-8 analog KC biomarkers were strong predictors of chronic cystitis development (Hannan *et al.*, 2010). Conversely, treatment with immunosuppressive compounds prior to infection is sufficient to curtail this response and is protective against

UTI in this model. It is postulated that the severe collateral damage caused to mucosal surfaces by the immune cell infiltrate are responsible for disease sensitization (Hannan *et al.*, 2010).

1.2.2 Management strategies for recurrent and chronic UTI

In general, UTI can be treated readily with short course antibiotic therapy (~3-7 days). This is primarily due to their very high urinary concentrations and the widely accepted superficial nature of bladder infections (Nickel, 2007). The effectiveness of single-dose therapies attest to this, with most antibiotics capable of clearing 90% of episodes (Nickel, 2007). However, available data support three day, short course therapy for best outcome of acute, uncomplicated UTI management. In some cases recalcitrance occurs following therapy cessation, resulting in recurrent or persistent infections and warranting the application of additional agents. There are various strategies available to manage patients with recurrent UTI, including culture directed therapy, long-term, low-dose antimicrobial prophylaxis, and post-coital antimicrobial prophylaxis (Nickel, 2005; Barber *et al.*, 2013). During therapy, symptoms abate and bacterial loads in voided urine decrease. However, it has been shown that prophylactic strategies do not alter the long-term risk of recurrence, as infection rates return to pre-treatment levels following therapy (Nickel, 2005) and device associated organisms are rarely cleared (Warren, 2001). In 2007 Conway *et al.* showed in a retrospective study that antimicrobial prophylaxis did not reduce risk of recurrence after initial infection, and actually increased the risk of producing resistant organisms. Problems such as these question the effectiveness of antimicrobial prophylaxis in managing device-related and recurrent infections, and whether the benefits of such treatments outweigh the risk of promoting resistance.

1.2.3 Issues with antibiotic therapy strategies and resistance

In general, UTIs receive little recognition in the infectious disease research community. This is likely due to the perception that although common, UTI pose little individual risk of complication. However, issues with UTI extend far beyond individual morbidity. As

they are one of the largest contributors of antibiotic prescription (Daneman *et al*., 2011), it is not unreasonable to recognize UTI as one of the greatest driving forces behind antibiotic resistance (Johansen *et al*., 2006). Unsurprisingly, countries with a higher consumption of antimicrobials have a higher incidence of resistance (Mackenzie *et al.*, 2007). This is reflected clinically, and despite their historic success in ameliorating acute cases, antibiotic therapy failure for UTI is on the rise (Pallett *et al.*, 2010; Gupta *et al.*, 2001). For example, current first-line empiric therapy has largely included the use of trimethoprim-sulfamathoxazole (co-trimoxazole). However, this agent is associated with a high resistance potential, and communities are experiencing unparalleled rates of highly resistant and untreatable strains (Manges *et al.*, 2001). Physicians have responded to this all-too-common problem by turning to fluoroquinolones for relief (Hooton, 2003). This practice has in turn resulted in increasing resistance rates against this important drug class (Johnson *et al.*, 2008).

Bacteria are able to take advantage of antibiotics to increase *de novo* mutation frequency and resistance, but are also able to enhance the acquisition of foreign resistance and virulence elements (Blazquez *et al.*, 2012). The impressive plasticity and adaptability of these organisms is realised when considering the multiple approaches used to increase their chance of surviving lethal antibiotic dosing. For example, antibiotic stress often results in the egress of mobile genetic elements such as IS-, phage-encoded virulence genes, integrating conjugative elements and phage-related pathogenicity islands, permitting their transfer to new organisms via horizontal gene transfer (HGT) (Schreiber *et al.*, 2013; Beaber *et al.*, 2004). Generally, mobilization is accomplished through the activation of the ubiquitous, general stress response system SOS, which is involved in double-strand DNA break repair. This results in the transcription of integrase, and cleavage of phage repressors via activated RecA and subsequent egress of mobile elements (Cambray *et al.*, 2011). However, antibiotic stress in organisms lacking SOS response systems can also potentiate transformation through the establishment of complement-type states and release of DNA via *in trans* allolysis (Prudhomme *et al.*, 2006). These processes could hypothetically contribute to the increasing frequency of recurrent UTI that has been observed since the widespread use of antibiotics.

Activation of SOS, and other general stress response systems such as RpoS results in low-fidelity PolIV expression, depletion of MutS and a reduction in mismatch-repair activity which induces hypermutator phenotypes (Gutierrez *et al.*, 2013; Kahrstrom, 2013). Beta-lactam, fluoroquinolones, aminoglycosides, tetracyclines and chloramphenicol all activate these systems to some degree in most organisms (Gutierrez *et al.*, 2013; Kahrstrom, 2013; Foster, 2007; Baharoglu and Mazel, 2011). Pathogens appear to have adapted to antibiotic stress by increasing their genetic flexibility, which results in acquisition of resistance through a number of mechanisms. Concentrations of antibiotics several hundred times lower than the MIC can enrich resistant mutants and result in development of *de novo* resistant strains (Gullberg *et al.*, 2011). Additionally, the acquisition of multiple copies of resistance elements can be accomplished through fluoroquinolone-dependent homologous recombination and homologous, redundant expansion of resistance determinants (López *et al.*, 2009). The importance of such genetic changes extends beyond the test tube, and has been shown to promote resistance and epidemic hospital infections (Hocquet *et al.*, 2012). Perhaps most worrisome is that these changes are cross-adaptive; that is, one antibiotic is capable of improving resistance against the same and multiple agents. In many cases, this occurs through the antibiotic-dependent expression of endogenous efflux pumps which indiscriminately clear noxious agents from the intracellular environment (Mc Cay *et al.*, 2010). Sub-inhibitory antibiotic treatments also have the capacity to increase tolerance through slight, stepwise changes to MICs often resulting in cross-resistance (Kohanski *et al.*, 2010). This is in contrast to highly-resistant mutants that appear spontaneously through selection and demonstrate little-to-no cross-resistance (Kaufmann and Hung, 2010). These problems are often recurring themes in the prophylactic management of recurrent UTI, as antibiotics are present throughout the course of dosing at concentration below the therapeutic threshold.

1.2.4 The importance in characterizing recurrences: relapse versus re-infection

Recurrences in the absence of resistance are believed to occur via one of two ways. The first involves re-inoculation of the bladder with organisms derived from the

gastrointestinal environment (re-infection). Indeed, transfer of organisms via the fecal-perineal-urethral route is common and supported with genetic evidence (Yamamoto *et al.*, 1997). These types of infection are identified using genome sequencing, designating new pathogens through strain variation from previous infection episodes. When re-infected patients are followed over a period of three months with daily urine and periurethral cultures, 75% of the strains causing recurrences were found to originate from the gut (Nickel, 2007; Chen *et al.*, 2013). Although it can be concluded that re-inoculation occurs in the vast majority of cases, it is also postulated that uropathogens can persist in dormant states within the urinary tract from previous infections, even with antibiotic intervention, and relapse (persistent infection) (Schilling *et al.*, 2002). In this manner, organisms may be present within the urinary mucosa but not detected by standard culturing techniques. Evidence supporting this hypothesis is also generated through sequencing, which delineates genetically identical organisms from previous infection episodes (same-strain recurrent UTI) (Jantunen *et al.*, 2002). In addition to increasing the risk of recurrence, these intracellular communities may explain other diseases whose aetiology remains unknown such as interstitial cystitis and overactive bladder. This is supported in murine studies which demonstrate animals resistant to antibiotic-clearance present with micro-abscesses in their urothelial tissue (Hannan *et al.*, 2010), albeit these findings do not disprove re-inoculation of the same organism from the gut.

Although it might seem trivial, differentiating infection sources is critical for outlining the problem of recurrence, and in designing an appropriate management strategy. Continuous re-inoculation of pathogens from the gastrointestinal tract could theoretically be curtailed through alterations in hygienic practices, but studies have shown that cleanliness of the genitalia alone is not to blame for females suffering from repeated UTI. On the other hand, sexual activity is influential by depositing urethral and vaginal organisms into the bladder (Stauffer *et al.*, 2004). In general, application of prophylactic antibiotics in this patient cohort is unwarranted, and may actually make conditions worse through the selection of resistant organisms (Kodner *et al.*, 2010). However, physicians still prescribe long-term extended use prophylactic therapy for such cases (Enzler *et al.*, 2011). Alternatively, and especially in situations where sexual activity is believed to be a

major driving factor for recurrence, post-coital or patient directed therapies have been met with success (Kodner *et al.*, 2010; Nickel, 2005). In this manner, the antibiotic is only supplied when it is perceived to be required, but depending on the frequency of sexual encounters, this could accumulate to be a lot of antibiotic use over time, all with consequences for the organisms and patient. In the case of persistent UTI, recurrences are thought to be driven by deficient host immune status or enhanced pathogen virulence, and long-term, prophylactic therapy is relied upon to decrease pathogen burden while ameliorating symptoms. However, whilst the desirable outcome of decreased symptoms and ameliorating infection can be achieved, breakthrough infections still occur and the treatment may be forcing bacteria to become more recalcitrant within the bladder, as will be discussed later.

1.3 PATHOGENIC MECHANISMS OF COMMON UROPATHOGENS

1.3.1 Pathogenesis of Gram-negative UTI

Although host factors certainly contribute to infection risk and severity, it is generally the virulence traits of pathogen which dictates disease progression. Enteric bacteria are the leading cause of UTI, the foremost of which is uropathogenic *Escherichia coli* (UPEC) which represents the cause of 75-90% of uncomplicated infections (Laupland *et al.*, 2007). *E. coli* is a Gram-negative, facultatively anaerobic, non-sporulating, rod shaped organism which is also a normal constituent of the gut microbiota. These traits, in addition to its resourcefulness in utilizing a diverse array of carbon sources, rapid replication rate and ability to survive in a wide variety of unfavourable environments are all factors that make this organism an important uropathogen. In addition to its high incidence, UPEC are often associated with recurrence and chronic lower tract infection (cystitis), necessitating application of long-term, prophylaxis management. The ability of UPEC to initiate such deep-rooted and stubborn infections is attributed to the wide array of virulence factors at its disposal (Norinder *et al.*, 2012). While genetic studies of organisms isolated from human subjects have assisted in the search for critical uropathogenic virulence traits, murine models of disease which have been instrumental in elucidating the complicated interplay between host and pathogen.

Murine models provide an ideal stage with which to study uropathogenesis. Mice are readily inoculated, easily monitored in time with urinalysis, and flexible with regards to the wide array of established genetic backgrounds which are available (Hung *et al.*, 2009). The biology of their urinary tracts, methods of urine voiding and host defense strategies are analogous to those found in humans. Prior to initiating infection, UPEC must ascend the urethra against urine flow in order to access the bladder. Deficiencies in this aspect of host defense are common in incontinent patients, and predispose to recurrent cystitis (Raz *et al.*, 2000). In murine models of UTI, uropathogens are directly inoculated into the bladder, effectively subverting this aspect of host defense. Rapid

adhesion to the bladder mucosa is essential to avoid clearance during urine voiding (Thomas *et al*., 2002).

The urinary tract is characterized by a pseudostratified epithelial layer containing the transitional epithelium, with the bladder covered with a single layer of unique, terminally differentiated superficial umbrella cells (urothelium) (Wu *et al.*, 2009). Superficial umbrella cells are coated in a plaque of uroplakin which constitutes the asymmetrical unit membrane (AUM), and consists of four proteins, UPIa, UPIb, UPII, and UPIIIa, in addition to the extracellular proteoglycan mucin layer (Wu *et al.*, 1994). Together, the role of the AUM is to provide structural support to superficial umbrella cells during times of bladder distension as well as regulating membrane permeability (Sun *et al.*, 1996). These urothelial components are also thought to play an integral role in preventing intimate bacterial contact with the host. This is achieved through the negative charge imparted by the highly sulfated and carboxylated glycosaminoglycans of the AUM (Parsons *et al*., 1990). However, these defenses can be overcome by UPEC which express type 1 fimbriae, capable of binding mannosylated residues of the uroplakin UPIa using the tip adhesin FimH (Connell *et al.*, 1996; Xie *et al.*, 2006). Type 1 fimbriae are demonstrated to be critical to establishment of UTI and found in 80% of UPEC strains (Buchanan *et al.*, 1985; Keith *et al.*, 1986), and are highly upregulated in the urinary environment (Gunther *et al.*, 2001). Type 1 fimbriae are phase variable, and the presence of both fimbriated and afimbriated organisms contributes to UTI in adherent and planktonic phases respectively, with both states of colonization important for pathogenesis (Lim *et al.*, 1998). The production of highly mannosylated Tamm-Horsfall protein (THP - uromodulin) in the urine is a host defense component which capitalizes on the critical nature of type 1 fimbriae to UPEC infection, by binding and sequestering organisms from the host surface (Pak *et al*., 2001). On the other hand, it has been suggested that in some cases, THP can act as a substrate to which the UPEC adhere, thereby enhancing the risk of infection (Hawthorn *et al.*, 1991)

Once UPEC have accessed the urothelial surface, they rapidly invade superficial umbrella cells. This process is critical to the establishment of acute infection, and is thought to be mediated by the interaction of type 1 fimbriae with UPIa as it is the primary target of FimH. Binding then results in phosphorylation of the uroplakin receptor complex which

triggers invasion (Martinez *et al.*, 2000; Zhou *et al.*, 2001; Thumbikat *et al.*, 2009; Wang *et al.*, 2009). Internalization is mediated by the activation of Rho GTPases which triggers actin rearrangement and UPEC engulfment via a zippering mechanism (Martinez and Hultgren, 2002). Upon accessing the cytoplasmic environment, UPEC begin to proliferate rapidly, forming large, multicellular aggregates dubbed intracellular bacterial communities (IBCs) (Anderson *et al.*, 2003). Although IBC formation has been observed in several species of *Enterobacteriaciae*, Gram-positive organisms do not appear to possess this pathogenic mechanism. These unique bacterial aggregates do not form in undifferentiated urothelial cells, making study of this process *in vitro* difficult. It is believed that the dense actin networks within undifferentiated cells (such as those found in immortalized lines) hinder this aspect of UPEC pathogenesis as actin destabilization agents induce the formation of IBC-like bodies *in vitro* (Mulvey *et al.*, 2001; Berry *et al.*, 2009; Eto *et al.*, 2006).

Within this intracellular niche, UPEC are resistant to both the host immune system and antibiotics. This has been confirmed *in vivo*, as UPEC reservoirs in murine bladder tissues were not eradicated against a panel of 16 antibiotics representing several distinct drug classes (Blango and Mulvey, 2010). Generally, IBC formation begins immediately following invasion, and continues until 16-24 hours post infection, with the majority of UPEC found intracellularly at 12 hours post inoculation (Mulvey *et al.*, 1998). During these later time points, UPEC undertake a filamentous morphology, fluxing away from the core IBC into neighbouring cells or emerging from the apical surface of dying urothelial cells (Justice *et al.*, 2004). The process of umbrella cell evacuation also coincides with exfoliation of this urothelial layer (Mulvey *et al.*, 1998; Justice *et al.*, 2006). This host immune mechanism is thought to expel adherent and IBC contained UPEC through urination, which is frequent during infection. As umbrella cells are lost, basal elements of the urothelium differentiate to fill their void. These pseudo-differentiate cells might hinder further rounds of UPEC invasion and IBC formation, and are thought to contribute to pathogen clearance in mice which spontaneously resolve infection (Justice *et al.*, 2004). However, it is possible that UPEC are capable of invading distal layers of the urothelium through filamentation, resulting in formation of quiescent intracellular reservoirs (QIRs) which could feasibly contribute to future episodes of UTI

(Mysorekar and Hultgren, 2006). The dynamic interplay between host mucosal immunity and UPEC pathogenesis represents an elegant example of a co-evolutionary process.

1.3.2 Pathogenesis of Gram-positive UTI

Staphylococcus saprophyticus is the second leading cause of uncomplicated UTI and is implicated in approximately 10-15% of cases, affecting roughly 1 million women in the US alone each year (Schneider and Riley, 1996). It is a Gram-positive, coagulase negative uropathogen which is identified clinically on the basis of endogenous novobiocin resistance and lack of hemolysin activity (Raz *et al.*, 2005; Mctaggart and Elliot, 1989). Compared to UPEC, the mechanisms involved in *S. saprophyticus* pathogenesis are not well understood. However, like *E. coli*, the major reservoir for this uropathogen is the gastrointestinal tract and perineal skin (Latham *et al.*, 1983). The majority of infections caused by *S. saprophyticus* occur in young, sexually active women, with colonization more frequent during the summer and fall (Wallmark *et al.*, 1978).

A high infection frequency is likely due to the wide array of virulence factors at *S. saprophyticus'* disposal that grant it tropism to the urogenital tract, specifically the kidneys (Kline *et al.*, 2010). Reports have placed *S. saprophyticus* as causing ~13% of upper UTI, a higher incidence than that communicated for other uropathogens (Hedman and Ringertz, 1991). Urease is highly expressed in this organism and appears to be an important virulence trait, resulting in more severe infection when present (Gatermann *et al.*, 1989). Some other important factors include a fimbrial surface-associated lipase (Ssp), lipoteichoic acid, hemagglutinin/autolysin/adhesin fibronectin binding protein, uro-adherence factors A and B (UafA/UafB), and a surface-associated collagen-binding protein (SdrI) (Kline *et al.*, 2010; Matsuoka *et al.*, 2011; Gatermann *et al.*, 1992; Sakinc *et al.*, 2005; Sakinc *et al.*, 2006). Of these, the uro-adherence factors appear to play the most critical role in pathogenesis, with adhesion greatly reduced in their absence (Kuroda *et al.,* 2005). Despite their importance, the ligand for these adhesins is currently unknown. To complicate matters further, murine infection models for investigating pathogenic mechanisms of *S. saprophyticus* cystitis are not ideal as Uaf adhesins do not interact well with the murine urothelium, indicating the absence of ligand in this

organism (King *et al.*, 2011). However, *S. saprophyticus* is still capable of colonizing the kidneys of mice for extended periods of time, and SdrI and Ssp are important for persistence in this organ (Kline *et al.,* 2010). Although IBC formation is not observed in Gram-positive uropathogens, *S. saprophyticus* is capable of internalizing into bladder cells, presumably representing an important step in pathogenesis and persistence (Szabados *et al.*, 2008). Unlike *E. coli*, the mechanisms for *S. saprophyticus* internalization are not characterized, but are thought to be distinct from those of other staphylococci (Szabados *et al.*, 2008). Kidney invasion has not been investigated despite the importance of this organ in *S. saprophyticus* uropathogenesis and persistence. This forms a component of studies performed in this thesis.

Whole genome analysis has revealed an abundance of open reading frames (ORFs) important for osmoregulation and ion transport, giving this organism the unique ability to persistently colonize the urinary tract despite lacking the extensive arsenal of virulence determinants found in *S. aureus* (Kuroda *et al.* 2005). *S. saprophyticus* carries a capsular element which appears to have been acquired through HGT due to its lower than average GC content. Characterization of this capsule has demonstrated an important role in immune evasion through resistance to complement-mediated opsonophagocytosis by neutrophils (Park *et al.*, 2010). Encapsulation appears to come at a price, as its presence decreases the interaction of Uaf proteins with cellular targets resulting in a reduced adherence potential.

Approximately 10% of *S. saprophyticus* UTI will result in recurrent infection following antimicrobial therapy (Fowler *et al*. 1981). Although re-infection could explain recurrence, intracellular organisms have been implicated in persistent colonization and re-emergent infections (Hunstad and Justice, 2010; Szabados *et al.*, 2008). Furthermore, *S. saprophyticus'* propensity to colonize medical devices resulting in recalcitrant infections is reminiscent of other coagulase negative staphylococci (Lang *et al.* 1999). Often, treatment of colonized devices involves removal and stringent antimicrobial therapy. Device replacement of chronically implanted individuals can occur quite frequently, greatly increasing patient morbidity and decreasing quality of life.

1.4 MECHANISMS OF BACTERIAL PERSISTENCE AND ANTIBIOTIC TOLERANCE

In addition to achieving persistence in the urinary tract through pathogenic mechanisms, infecting organisms must also be able to withstand exposure to antibiotics. Bacterial survival following appropriate therapy is normally attributed to genetically resistant organisms; however, nearly all species also possess transient, phenotypic, multidrug-tolerant (MDT) members called persister cells, named for their capacity to persist despite exposure to a lethal antibiotic dose. Although the evidence has been largely circumstantial, persisters are thought to play a role in treatment failure and in further pathogenesis of chronic diseases such as cystic fibrosis, recurrent UTI and tuberculosis (Goneau *et al.,* 2014; Blango *et al.*, 2010; Keren *et al.*, 2011; Mulcahy *et al.*, 2010). The role of persisters in such infections may largely be overlooked as they are not tested for during routine clinical microbiology diagnostic procedures.

1.4.1 The nature of MDT

Since their discovery by Joseph Bigger in 1944, persisters have remained largely unclassified and poorly characterized (Bigger, 1944). However, they are generally believed to comprise a fraction of the overall population (~0.01%), and are thought to have suppressed metabolic activity and replicative capacity which prevents antibiotic activity (Lewis, 2010). The bactericidal nature of antibiotics is thought to be mediated through the corruption of cellular machinery, preventing them from performing their normal functions required for homeostasis. Genetic resistance is achieved by preventing this interaction, either by altering target antibiotic binding sites, destroying or modifying the drug, or active removal via cellular efflux (Wright, 2005). Conversely, it is postulated that reduced activity would confer a protective phenotype (Lewis, 2010). Thus, physiological changes are thought to confer MDT in persisters.

A consensus regarding the nature of MDT has yet to be reached, with two scientific camps disputing whether persisters represent truly dormant bacterial variants or simply a slow growing sub-population of the greater culture (Goneau *et al.*, 2014; Balaban *el al.*,

2004; Wakamoto *et al.*, 2013; Johnson and Levin, 2013). Initial experiments involved sorting dormant organisms from the remaining, metabolically active population. Importantly, these sorting experiments successfully demonstrated that dormancy correlated with persistence, as dormant populations had 20-times more persisters than normally growing populations (Shah *et al.*, 2006). However, the authors also conclude that dormancy alone is not necessary and sufficient to explain persistence. In addition, most persisters demonstrate levels of mRNA which can be extracted and amplified. With its short half-life, the presence of mRNA is indicative of active transcription, suggesting at least some level of physiological activity is present in persisters (Hu *et al.*, 2000). Distinguishing between these two proposed models is critical in evaluating new therapeutic targets focused at persister eradication and the amelioration of chronic disease. The issue will lie in either learning how to potentiate antibiotics against already active persisters, or figuring out how to 'wake them up' from dormancy to enable killing (Allison *et al.*, 2011[a]; Allison *et al*., 2011[b]). The presence of antibiotics within even slowly replicating persisters may potentiate hypermutator phenotypes, acquisition of resistance traits and establishment of breakthrough infections. Organisms in persister-related infections such as cystic fibrosis are capable of mutagenesis and increasing their persister fraction >100-fold from pre-antibiotic levels (Mulcahy *et al.*, 2010). Since persister traits are under antibiotic selective pressure, it is possible that organisms with large persister fractions may be refractory to antibiotic therapy in severe infections such as sepsis where rapid, successful antimicrobial intervention is critical to patient survival.

1.4.2 Mechanisms and genetics of persister formation

Although MDT is conferred through physiological changes, genetics certainly dictate the dynamics of persistence. Formation is believed to be driven by two processes, with Type I persisters classified as non-growing variants which are enriched naturally during late exponential and stationary phases (Luidalepp *et al.*, 2011), and slowly-growing and dividing Type II persisters whose formation occurs stochastically during exponential phase due to random fluctuations in transcriptional and translational noise (Balaban *et al.*, 2004; Balaban, 2011; Kussel and Leibler, 2005). Enrichment of Type II persisters can

occur through various stressors such as antibiotics, resulting in fractions that can range from 10-100% of the overall population (Kwan *et al.*, 2013). Such stressors generally result in MDT through the activation of various toxin/antitoxin (TA) systems, which are believed to provide a common 'emergency stop' response mechanism to halt environmental stress (Dörr *et al.*, 2010; Kint *et al.*, 2012; Moyed *et al.*, 1983; Allison *et al.*, 2011). This is an intriguing model as TA systems appear to be ubiquitous in prokaryotes (Grønlund and Gerdes, 1999; Schuster and Bertram, 2013). TA systems usually consist of a stable toxin protein, and a labile RNA or protein antitoxin which prevents toxicity. The toxin is involved in disruption of an essential cellular process, such as translation interruption via mRNA degradation or decoupling of the proton motive force and ATP production (Gerdes *et al.*, 2005). Mutagenesis studies were the first to identify the role of TA systems in persister formation, identifying alterations in the *hipBA* locus as predisposing to persistence (Moyed *et al.*, 1983). Characterization of the mutant strains, specifically the *hipA7* gain of function variant, revealed that decreasing binding affinity of the toxin (HipA) for its cognate antitoxin (HipB) was responsible for increased persistence and drug tolerance (Korch and Hill, 2006). Since identification of this TA system's role in persistence, numerous other TA pairs have been implicated including YafQ/DinJ, RelE/RelB, MazF/MazE, TisB/IstR-1 and others, with overproduction of RelE increasing persistence as much as 10,000-fold (Keren *et al.*, 2004). The wide array of TA pairs involved in persistence demonstrates the redundancy of these systems, suggesting no single drug will be capable of overcoming all these bacterial fail-safes. Additionally, different mechanisms of onset might indicate that persister cells are distinct, and represent a heterogeneous sub-population with unique susceptibilities to antibiotics (Allison *et al.*, 2011). In this sense, MDT might not be a function of individual persister states, but rather the combined influence of multiple persisters with unique susceptibility profiles which contributes to the tolerance of the entire population.

Activation of TA systems is intimately connected to induction of the SOS response, which is also a common mechanism for the establishment of genetic resistance. In fact, cells appear to react to stress by either: i) activating stress response elements in hopes of resisting it, or ii) converting part of the population to persister states through the activation of TA modules. Together, these represent a bet-hedging strategy which permits

survival of at least a fraction of the population (Dörr *et al.*, 2010). Dynamic heterogeneity within individual cells determines which outcome is selected, and involves the SOS-inducible Lon protease and subsequent degradation of antitoxin elements (Maisonneuve *et al.*, 2011). Several agents appear to induce persister formation in this manner, including DNA gyrase, transcription, translation, ATP synthesis and transpeptidase inhibitors (Dörr *et al.*, 2009; Miller *et al.*, 2004; Kwan *et al.*, 2013). In this way, antibiotics may be capable of propagating chronic and recurrent infections by continuously stimulating the formation of persisters.

Persister cells are also intimately linked to high-density growth states, such as those found during stationary phase, increasing in frequency from ~0.01% of the population during early-exponential phase to over 10% at later stages of growth (Tashiro *et al.*, 2012). Persister cells are also enriched in biofilms, where they are thought to contribute to antibiotic tolerance which can be up to 1000-times higher than their planktonic counterparts (Roberts and Stewart, 2005; Lewis, 2001). When found in biofilms, persister cells are not only refractory to antibiotic therapy, but also to immune detection and clearance (Lewis, 2007). In many cases, biofilm infections are deep-rooted, associated with medical devices or other complicated patient cohorts, and are incurable. Surprisingly, quorum sensing does not seem to contribute to formation, as the addition of spent culture supernatants containing high concentrations of quorum-sensing compounds did not appreciably increase the number of persisters isolated for *E. coli* (Lewis, 2007). Thus, the mechanisms underlying persister formation during these growth stages remains a puzzle.

1.5 HYPOTHESIS AND OBJECTIVES

It is hypothesized that low-dose antibiotic therapy will increase the risk of chronic and recurrent UTI through the modulation of both host and pathogen responses.

As outlined above, the management of patients with recurrent UTI remains a challenge due to pathogen re-emergence, resistance and the risk of potentially fatal sepsis. Strategies typically rely on the long-term application of low-dose antibiotics as a means of discouraging pathogen re-propagation within the urinary tract while avoiding the unfavourable side-effects of prolonged exposure at higher doses. Unfortunately, this dosing regimen potentiates the presence of antibiotics at sub-therapeutic and sub-inhibitory concentrations, which appear to influence microorganisms in unexpected ways, potentially causing unpredicted changes to virulence traits including adherence, invasiveness, and persistence. These changes may be reflected in the questionable benefits of prophylaxis which do not seem to alter long-term recurrence risk while also often failing to resolve established infection (Nickel, 2005; Williams and Craig, 2009). Bacterial changes in response to therapy may also explain cases of antibiotic failure despite the absence of resistant organisms (Mulcahy *et al.*, 2010; Levin and Rozen, 2006).

This thesis explores these clinical observations in the context of inappropriate antimicrobial therapies including prophylaxis by evaluating whether they have the capacity to negatively influence host-pathogen interactions during UTI. Agents used throughout include ciprofloxacin, ampicillin, and gentamicin, for their extensive clinical use and distinct targeting mechanisms. Specifically, the fluoroquinolone antibiotic ciprofloxacin targets and corrupts DNA topoisomerases II and IV by selectively inhibiting ligase activity while leaving nuclease functionality unhindered (Drlica and Zhao, 1997). Constant nuclease activity combined in the absence of ligation introduces double-strand DNA breaks resulting in fragmentation. Ampicillin is a beta-lactam antibiotic which irreversibly binds to and inhibits the transpeptidase enzyme (Waxman

and Strominger, 1983). Continued autolysin activity at the cell wall results in lysis. Lastly, gentamicin functions by binding to and inhibiting the 30S subunit of the bacterial ribosome. Interruption of protein synthesis and production and intracellular accumulation of corrupted protein products results in cell death (Davis, 1987). All three agents share characteristic broad-spectrum activity and are bactericidal.

Objectives:

(1) Examine the effects of sub-inhibitory antibiotics on uropathogen virulence

Only recently has the capacity of antibiotics to modulate aspects of bacterial virulence been explored, and never in the context of uropathogens. As UTI is a major reason for antibiotic prescription and results in complicated and debilitating disease in hundreds of millions of women worldwide, it is critical to understand how pathogenesis is affected during treatment. Thus, the ability of sub-inhibitory antibiotics, targeting distinct cellular processes, was assessed for virulence-modulating capacity on both the phenotypic and gene expression levels.

(2) Demonstrate the influence of sub-inhibitory ciprofloxacin on uropathogenesis in a murine model of UTI

Although transcriptomic studies are abundant, none have explored the changes in pathogenesis which follow sub-inhibitory treatment. Mouse models of UTI are a well established experimental system which permit the investigation and characterization of antibiotic-induced changes *in vivo*. Using this model, experiments were conducted to demonstrate that *in vitro* alterations in virulence factor expression are mirrored *in vivo* and influence the ability of the host to clear infection.

(3) Understand the ability of uropathogens to persist despite adequate antibiotic intervention during infection

The role of persister cells in chronic diseases is not well documented, and the nature of MDT is not clearly defined. However, these unique physiological variants are thought to play an important role in recurrence risk following UTI antibiotic management. Thus, the importance of persister states in recurrent UTI were investigated using clinical isolates. In addition, the contribution of dormancy toward MDT in uropathogens was explored, and took into account the dynamics of antibiotic-dependent persister cell induction as well as maintenance by analyzing both Type I and Type II persister fractions.

CHAPTER TWO

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 BACTERIAL STRAINS AND CULTURE CONDITIONS

All media were acquired from BDH (Toronto, ON) or Becton and Dickinson (BD; Oakville, ON), with supplements from BDH or Sigma (Mississauga, ON). Bacterial strains were cultured from -80°C stocks and routinely grown at 37°C using media and conditions conducive to each strains' growth requirements. Unless stated otherwise, staphylococci and *Escherichia* were grown using lysogeny Broth (LB), supplemented with antibiotics when appropriate. Minimal inhibitory concentration (MIC) determination and antibiotic susceptibility experiments were performed at 37°C in cation-adjusted Mueller Hinton (MH) II medium (BDH). Human pooled urine (HPU) was also used as a growth substrate. Preparation involved pooling the urine from at least three volunteers, filter sterilization using a 250 mL Nalgene Rapid-Flow vacuum filter unit equipped with 0.22 µm PES membrane (Thermo Scientific, Ottawa, ON), and pH adjustment to 6.5. Enumeration of bacterial colony-forming units (cfu) was performed using LB agar (Bacto-agar; BD) plates prepared as per the manufacturer's instructions and supplemented with antibiotics when required. All experiments made use of cultures grown in 15 mL BD Falcon tubes, excluding those involving murine infection models which made use of 100 mL Erlenmeyer flasks. Cultures were grown statically unless stated otherwise. If shaking was implemented it was done so at 200 rpm.

All strains are listed in Table 2.1. Both *E. coli* CFT073 [isolated from a woman with acute pyelonephritis (Kao *et al.*, 1997)] and *S. saprophyticus* 15305 [UTI isolate (Shaw *et al.*, 1951) - type strain] were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), while the UPEC clinical isolates *E. coli* TOP277, TOP263, TOP379, TOP344, TOP345, PUTS277, PUTS278, PUTS1127, PUTS1236 and UTI89 (att_{HK022}*::COM-GFP*; kanamycin-resistant, Kan^R) were kindly provided by Dr. Scott Hultgren (Washington University School of Medicine, St Louis, MO). Briefly, each strain was derived from a distinct patient presenting with recurrent UTI. Patients were sampled over time and organisms isolated from each infection episode were sequenced

Table 2.1 Bacterial strains used in this study

Strain (type)	Source
S. saprophyticus strains	
15305	ATCC[a]
15305:C1	Toshiko laboratory
7108	Hultgren laboratory
E. coli strains	
CFT073	Our laboratory
UTI89 att_{HK022}*::COM-GFP:Kan*^{R}	Hultgren laboratory
UTI89 *pANT4::GFP*	Hultgren laboratory
UTI89 Δ*fimS* (Δ*fimA-fimH*)	Hultgren laboratory
UTI89 Δ*fimH*	Hultgren laboratory
UTI89 Δ*recA*	Justice laboratory
UTI89 $lexA_{T355G}$	Justice laboratory
TOP277	Hultgren laboratory
TOP263	Hultgren laboratory
TOP379	Hultgren laboratory
TOP344	Hultgren laboratory
TOP345	Hultgren laboratory
PUTS278	Hultgren laboratory
PUTS1127	Hultgren laboratory
PUTS1236	Hultgren laboratory
PUTS277	Hultgren laboratory

[a] American Type Culture Collection (ATCC)

(Garofalo *et al.*, 2007). If repeat recurrences were caused by the same strain the isolate was assigned an SSR designation. Conversely, infections caused by a strain which varied from previous infections were designated as AI. SOS-deficient strains *E. coli* PAS0209 (Δ*recA*) and PAS0211 ($lexA_{T355G}$; also referred to as $lexA_{G85D}$ or Ind- mutant) derived from the UTI89 background were provided by Dr. Sheryl Justice (The Research Institute at Nationwide Children's Hospital, Columbus, OH). The acapsular *S. saprophyticus* C1 strain derived from the 15305 background was provided by Dr. Toshiko Ohta (Graduate School of Comprehensive Human Science, University of Tsukuba, Tsukuba, Japan).

2.2 ANTIBIOTIC SUSCEPTIBILITY TESTING

Susceptibility testing was performed using the Kirby-Bauer method as per standard Clinical and Laboratory Standards Institute (CLSI) guidelines (2007). Briefly, strains were grown in MH broth overnight in a shaking incubator (200 rpm) at 37°C. Cultures were diluted five-fold in phosphate buffered saline (PBS; pH 7.4) and streaked across the entire surface of an MH agar plate using a cotton-tipped swab. Ciprofloxacin (CIP-5 µg), ampicillin (AMP-10 µg), and gentamicin (GEN-10 µg) test discs (VWR, Mississauga, ON) were added each to one third of the plate. Zones of growth inhibition (ZOI - mm) around each disk were determined following overnight incubation. The ZOI for each strain was compared to referenced standards to determine the clinically relevant category of susceptibility for each antibiotic.

2.3 MINIMAL INHIBITORY CONCENTRATION DETERMINATION

The MICs to ciprofloxacin, ampicillin, and gentamicin (Sigma) was determined for all organisms using the broth microdilution technique outlined by the CLSI (2007). Briefly, strains were grown in MH broth overnight in a shaking incubator (200 rpm) at 37°C. Cultures were then inoculated into MH broth containing various two-fold dilutions of antibiotics and grown statically for 24 hours in a 96-well plate. The MIC was regarded as the last well demonstrating growth based on turbidity. Experiments were carried out with

at least three independent replicates. MIC quality control was performed using control organisms recommended by the National Committee for Clinical Laboratory Standards with known MIC values for antibiotics used in this study. Optimal sub-inhibitory antibiotic dosing strategies were evaluated to determine concentrations which confer phenotypic effects without perturbing growth. Although a range of concentrations were considered, 1/4MIC levels were the maximal dose that organisms could tolerate without loss of fecundity. Therefore, this dose was used throughout unless stated otherwise.

2.4 MICROSCOPE SLIDE ADHERENCE ASSAY

Overnight (24 hour) cultures of *S. saprophyticus* 15305 grown in HPU were diluted 100-fold into fresh HPU and incubated for three hours. Ciprofloxacin, ampicillin, gentamicin or vehicle were applied at 1/4MIC levels and incubated for one hour to permit establishment of physiological changes. Following this period, 100 μL of culture were transferred to the surface of uncoated glass microscope slides. Slides were covered and incubated for 30 minutes to permit organism adhesion. Experiments were conducted in duplicate. Slides were rinsed gently for 5-10 seconds with PBS to remove weakly adherent or unbound organisms, heat fixed, and stained for two minutes with crystal violet. The number of adherent *S. saprophyticus* 15305 was determined by enumerating the number of clearly defined organisms in at least five representative fields of view (FOVs) at a magnification of 1000x on an Axioskop microscope (Carl Zeiss, Toronto, ON).

2.5 STENT AND CATHETER ADHERENCE ASSAY

Overnight (24 hour) cultures of *S. saprophyticus* 15305 grown in LB were reconstituted 1000-fold in fresh media. After a three hour recovery period, 1 mL of culture was transferred to a microfuge tube containing 1 cm segments of either Foley urethral catheter (BARD Medical, Covington, GA) or InLay Optima® ureteral stents (BARD Medical). Ciprofloxacin, ampicillin, gentamicin, or vehicle were applied at 1/4MIC levels to the cultures, and incubated for one hour at 37°C. At this point, 10 μL of cultures were

extracted from each treatment group and dilution plated to obtain total population numbers (each sample served as its own control). Stents and catheters were removed, washed twice with PBS to remove loosely attached organisms, and placed in fresh microfuge tubes containing 1 mL of PBS. These tubes containing the washed stents and catheters were transferred to a Branson 1200 ultrasonic water-bath (Emerson, Danbury, CT) and sonicated for 10 minutes to liberate adherent organisms. Samples were extracted from each tube and dilution plated to determine the number of adherent *S. saprophyticus* 15305.

2.6 BIOFILM FORMATION ASSAYS

Since, ciprofloxacin, ampicillin and gentamicin are capable of inducing the SOS response, it is reasonable to postulate that observed antibiotic-dependent modulation of adherence and biofilm formation is driven through the activation of this regulon. This hypothesis was tested by comparing the biofilm-priming potential of antibiotics in wild-type and SOS deficient strains of *E. coli* UTI89. SOS-deficient strains consisted of PAS0209 (Δ*recA*) and PAS0211 ($lexA_{T355G}$ - has T-to-G change at position 355 of encoded by *lexA*; also referred to as $LexA_{G85D}$ or Ind mutant) derived from the UTI89 background. Both strains were utilized in order to compare and contrast the contribution of severe (Δ*recA*) and moderate ($lexA_{T355G}$) double-strand DNA break repair and SOS deficiencies.

Overnight (24 hour) cultures of *S. saprophyticus* 15305 or *E. coli* strains UTI89, PAS0209, and PAS0211 grown in LB were reconstituted 1000-fold in fresh media but not incubated further. The wells of a 96-well plate were prepared by loading with various sub-inhibitory concentrations of either ciprofloxacin, ampicillin, gentamicin, or vehicle in 100 µL of LB. Organisms were added at 10 µL per well, plates were sealed, then incubated for either 24 or 48 hours at 37°C. If incubation periods continued for longer than 24 hours, wells were washed and replaced with fresh LB containing the appropriate concentration of antibiotic. Following these incubation periods, wells were washed with PBS three times to remove planktonic and weakly adherent organisms, stained with crystal violet for two minutes, washed again with cold dH_20 and decolorized with 100 µL

70% ethanol. Biofilm abundance was determined by measuring the absorbance at 600 nm (OD_{600}).

2.7 SCANNING ELECTRON MICROSCOPY PROTOCOL

Samples examined using scanning electron microscopy (SEM) were prepared as described above (stent and catheter adherence assay). Once organisms had adhered and stent and catheter material had been washed, samples were exposed to a pre-fixative solution containing 2.5% glutaraldehyde (Sigma), 50 mM L-lysine monohydrochloride (Sigma), and 0.75% Ruthenium Red (Sigma), in 0.2 M cacodylate buffer (pH 7.4), as described by Williams *et al*. (2010). Material segments were soaked in pre-fixative for a period of 20 minutes, then transferred to a fresh pre-fixative solution excluding the 50 mM L-lysine for 24 hours, as per the protocol of Priester *et al.* (2007). Following pre-fixative treatment, samples were rinsed in cacodylate buffer three times for 10 minutes, placed in a 2% solution of osmium tetroxide (OsO_4; Sigma) in cacodylate buffer for two hours, then rinsed with cacodylate buffer three times for 10 minutes. Following rinsing, stent and catheter segments were placed in increasing concentrations of ethanol, from 70% to 95% to 100%, three times for 20 minutes each. Samples were placed in a desiccator following critical point drying for at least 24 hours or until imaged.

Imaging of samples was conducted at Surface Science Western (Western University, ON). Prior to imaging, stent and catheter segments were coated using platinum sputtering. All samples were imaged using a Hitachi S-4500 field emission SEM with Quartz XOne EDX system (at 10 kV. Energy dispersive X-ray (EDX) spectroscopy was used to determine the elemental composition of surfaces in different treatment groups.

2.8 NEGATIVE STAINING AND ANALYSIS BY TRANSMISSION ELECTRON MICROSCOPY

Bacteria were allowed to absorb onto Formvar® (SPI-Supplies, Toronto, ON), or carbon-coated copper grids for one minute. Grids were washed in dH_2O and stained with 1%

aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for one minute. Excess liquid was gently wicked off and grids were allowed to air dry. Samples were viewed on a JEOL 1200EX transmission electron microscope (JEOL USA, Peabody, MA) equipped with an AMT 8 megapixel digital camera (Advanced Microscopy Techniques, Woburn, MA) at 80 kV.

2.9 *IN VITRO* UROTHELIUM ADHERENCE AND INVASION PROTOCOL

2.9.1 Kidney and bladder cell maintenance

A498 kidney cells (ATCC HTB-44) were maintained in Eagle's minimum essential medium (EMEM; Gibco, Burlington, ON) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% L-glutamine (Gibco), 1% sodium pyruvate (Gibco), and 1% non-essential amino acids (Gibco). 5637 (ATCC HTB-9) and T24 bladder cells (ATCC HTB-4) were maintained in RPMI-1640 medium (Gibco) supplemented with 10% FBS and 1% L-glutamine. All cells were maintained in T75 flasks, in a 5% CO_2 tissue culture incubator at 37°C. Cells were routinely passed when confluency reached greater than 90%. This was accomplished by briefly washing with warm PBS followed by 0.05% trypsinization (Gibco) for 6-8 minutes at 37°C and cell dislodgement via gentle tapping. After trypsin treatment, cells were either maintained by reconstituting five-fold in a new T75 flask with fresh media, or 48 hours prior to infection, 2×10^4 cells were transferred to a 24-well plate and grown to confluency.

2.9.2 *In vitro* tissue culture infection protocol

Overnight (24 hour) cultures of *S. saprophyticus* 15305 or the acapsular C1 strain were grown in LB were reconstituted 100-fold in fresh supplemented EMEM for A498 kidney infections, or supplemented RPMI-1640 medium for 5637 and T24 bladder infections. Samples were grown statically for three hours at 37°C with relevant antibiotic treatments

at 1/4MIC or vehicle. A498, 5637, or T24 were maintained in 24-well plates and infected with pathogen when achieving at least 90% confluency (~1.1 x 10^5 cells). Prior to infection, cells were washed at least thrice with warm PBS, with warm, fresh media replaced after the final wash. Infections were conducted at a multiplicity of infection (MOI) of 25:1 for *S. saprophyticus* 15305 per host cell, and carried out for four hours in a bacterial cell culture incubator with 5% CO_2 at 37°C.

2.9.3 Determining adherence frequency

Adherence was determined by removing planktonic cells with two PBS washes. Each well was then replaced with fresh PBS, and cells lysed with the application of triton X-100 (Fisher Scientific, Ottawa, ON) solution at 0.1% for 15 minutes at 37°C. Dilution plating of the resulting lysate permitted enumeration of the combined 'adherent' and 'internalized' bacterial populations. As internalization occurred much less frequently than adhesion the relative contribution of intracellular organisms to the overall adherent population was negligible. Therefore, this value was taken to represent the adherent population. This was compared to the total population numbers to determine percent adherence.

2.9.4 Determining internalization frequency

Internalization frequency was determined using gentamicin protection assays. Cells were washed two times with PBS to remove planktonic organisms, with wells subsequently refilled with fresh media containing 10 µg/mL gentamicin. Samples were incubated for three hours in a bacterial tissue culture incubator with 5% CO_2 at 37°C in order to kill non-internalized organisms. Cells were then washed thrice with PBS, and lysed as described. The percentage of intracellular organisms was determined by spread plating 100 µL of lysate and comparing to total population numbers.

2.10 HEMAGGLUTINATION ASSAYS

Type 1 fimbriae and UafA adhesins readily agglutinate guinnea pig and sheep erythrocytes respectively, providing a means for the rapid quantification of surface expression following sub-inhibitory antibiotic treatment (Salit and Gotshclich, 1977; Kuroda *et al.*, 2005). This assay has the added benefit of directly measuring adhesin levels at the bacterial surface which is advantageous over other techniques involving RNA expression analysis which might not correlate with true protein expression levels. However, this benefit can also be influenced by false negatives when surface components interfere with the process of hemagglutination. This has been shown to occur when capsule is present (Kuroda *et al.*, 2005; Park *et al.*, 2010). Thus any observable changes in hemagglutination could presumably be due to UafA upregulation, or capsule downregulation. Therefore, the acapsular *S. saprophyticus* strain C1, derived from the 15305 background, was also utilized in addition to the well-characterized capsule-deficient strain 7108 (Park *et al.*, 2010).

2.10.1 Blood preparation

Alsevers guinea pig red blood cells (RBC) (Colorado Serum Company, Denver, CO) (*E. coli* hemagglutination) or sheep RBCs (Fisher Scientific) (*S. saprophyticus* hemagglutination) were swirled to mix prior to use. An erythrocyte suspension was created by diluting RBCs 10-fold in chilled PBS, inverting several times to mix to avoid lysis. Erythrocytes were washed by repeated pelleting (spinning in a clinical centrifuge for ~1-2 minutes) and resuspension in chilled PBS until supernatants were clear and colourless (usually 1-2 washes). Once clear, supernatant was aspirated, and RBCs were resuspended in ~7 mL PBS until OD_{640} of 2.0 was achieved. RBC suspensions were placed on ice until needed. Absorbance was measured using a Eon microplate spectrophotometer (BioTek, Winooski, VT).

2.10.2 Bacterial and plate preparation, and assay

E. coli UTI89, PAS0209, or PAS0211, and *S. saprophyticus* 15305, or C1 were reconstituted 1000-fold from overnight (24 hour) cultures into fresh LB media containing 1/4MIC of relevant antibiotics or vehicle. In addition to the wild-type strain, the acapsular *S. saprophyticus* C1 strain was used as a positive control for UafA-dependent hemagglutination. Organisms were grown in their respective treatment for four hours at 37°C. Following this period, bacteria were pelleted at 6500 rpm for three minutes and resuspended in PBS to OD_{600} of 1.0. 1 mL of the resulting suspension was transferred to a 1.5 mL microfuge tube, and centrifuged at 6500 rpm for two minutes using a Sorvall Legend Micro 21 centrifuge (Thermo Scientific) to pellet. Supernatants were then aspirated, and pellets resuspended in 100 µL of PBS.

V-bottom, 96-well plates were prepared by transferring 25 µL of PBS into each well. Additionally, % (w/v) a-methyl-D-+-mannopyranoside (Sigma) was also prepared in a second plate in a similar manner, and served as a negative control for type 1 fimbriae-dependent hemagglutination. In duplicate, 25 µL of each suspension was transferred to the first column of the plate, and serially diluted two-fold into each subsequent well to create a dilution gradient of organisms. 25 µL of the prepared blood suspension was added per well, and mixed by gently tapping the plate. Plates were then sealed, covered, and placed at 4°C for 2-3 hours prior to analysis. Titers were read by determining the last well to display RBC agglutination.

2.11 TYPE 1 FIMBRIAE PHASE VARIATION

The expression of type 1 fimbriae *fim* genes is finely regulated by environmental signals including growth phase, nutrient abundance and culture flow (Abraham *et al*., 1985). Combined, these signals dictate the percentage of fimbriated cells in the overall population, a process which is phase variable and associated with the inversion of a short DNA *fim*-switch element which grants the transcriptional machinery promoter accession. The proportion of *E. coli* UTI89 type 1 fimbriae in the phase ON and OFF orientation was assessed using PCR. Antibiotic-dependent induction of the SOS response has been

linked to adhesin upregulation in *S. aureus* (Bisognano *et al.*, 2000). Additionally, SOS-deficient *E. coli* UTI89 strains demonstrate decreased virulence potential in a murine model of UTI (Justice *et al.*, 2006). Importantly, type 1 fimbriae saturation occurs in static cultures, while shaking cultures are generally comprised of phase OFF variants. Therefore, shaking cultures were also taken advantage of to permit more sensitive identification of antibiotic-dependent phase switching by avoiding the saturation effects of static cultures. Organisms were incubated with antibiotic or vehicle for 4, 8, or 24 hours as previously described prior to phase analysis. At these time points, 1 µL of culture would be extracted, and used as DNA template during PCR reactions.

2.11.1 PCR mixes and protocols

PCR recipe included 5 µL of 10x PCR buffer, 2.5 µL of 50 mM $MgCl_2$, 1 µL of 10 mM dNTPs, 1 µL of 10 µM Phase Left Primer, 1 µL of 10 µM Phase Right Primer, 38 µL of dH_2O, and 0.2 µL Taq DNA polymerase (Thermo Scientific). The master mix was prepared for n-reactions by multiplying each reagent by n+1 (not template) and transferring 25 µL of the resulting mix to PCR tubes for amplification. PCR primers A (5'-GAGAAGAGGTTTGATTTAACTTATTG-3') and B (5'-AGAGCCGCTGTAGAACTGAGG-3') were used for amplification of the *fim* switch region as described by Roesch and Blomfield (1998).

PCR amplification protocol included the following steps: i) 95°C for 5 minutes, ii) 95°C for 1 minute, iii) 55°C for 1 minute, iv) 72°C for 1 minute. Steps ii) through iv) were repeated 35 times for amplification, followed by a final 72°C elongation step for 10 minutes. If samples were not immediately analysed, they were held at 4°C until required. Reactions were carried out on a MyCycler Thermal Cycler (Bio-Rad, Mississauga, ON).

2.11.2 Restriction digestion, gel electrophoresis, and ImageJ analysis to detect phase

PCR reactions were split into another tube, so as to permit one tube for restriction digest and one tube as an undigested control. 1 uL of HinfI (New England Biolabs, Whitby, ON) was added to each tube to be cut, and incubate at 37°C for two hours. 5 uL of each digest and 5 uL of the original PCR reaction on was run on a 2.0% agarose gel (in TBE with ethidium bromide added directly to gel once sufficiently cooled). The full PCR product is 559 base-pairs. HinfI digestion yields 70 base-pair and 489 base-pair products for Phase ON, while 200 base-pair and 359 base-pair products are observed for Phase OFF. The intensity of the 489 and 359 base-pair products were analyzed and compared via densitometric analysis using ImageJ software (http://rsb.info.nih.gov/ij/).

2.12 SOFT-AGAR MOTILITY ASSAY

Flagella are the driving force behind bacterial motility, and are generally suppressed as organisms' transition from planktonic to adherent states (Pesavento *et al*., 2008). The activity of flagella can be measured by assessing bacterial swarming over the surface of soft-agar plates. Soft-agar plates (0.3% agar) were prepared by either loading with 1/4MIC levels of antibiotic or vehicle. This concentration of agar was previously found to be ideal for investigating bacterial swarming motility (Croze *et al.*, 2011). *E. coli* UTI89, PAS0209, or PAS0211 were reconstituted 1000-fold in fresh LB without antibiotic, incubated at 30°C with shaking (200 rpm) for four hours , and adjusted to OD_{600} of 1.0 before being spot-plated onto the surface of antibiotic loaded or unloaded soft-agar plates. Organisms were also pre-treated with 1/4MIC levels of relevant antibiotics for 4 hours as described previously, and spot plated onto the surface of unloaded soft-agar plates. These approaches permitted analysis of how antibiotics influence the expression and activity of flagella in planktonic phase and surface-bound *E. coli*. Organisms were incubated for 24 hours at 30°C to allow the formation of a swarming zone on the surface of soft-agar plates. At this time, the radius of the swarming zone (mm) was measured for quantification of swarming motility.

2.13 MURINE INFECTION PROTOCOLS

Murine infections models (Hung *et al.*, 2009) routinely made use of 7-9 week old female C3H/HeN or C57BL/6 mice. The animals were obtained from Harlan laboratories (Harlan Sprague Dawley® Inc.; Indianapolis, IN) at 6-7 weeks of age, and allowed a 5-7 day rest period prior to experimentation as stress can have a significant impact on reproducibility. For this reason, use of mice greater than nine weeks of age was avoided. All animal studies were conducted in accordance to the Guide for the Care and Use of Laboratory Animals under the Animal Welfare Assurance number A3381-01, at the Washington University School of Medicine, St. Louis (St. Louis, MO). The Washington University School of Medicine Animal Study Committee approved all the experimental procedures described. Mice were housed in micro-isolator cages in a BSL2 barrier animal facility and fed autoclaved standard laboratory rodent diet (Purina) and autoclaved tap water *ad libitum*.

2.13.1 Bacterial and syringe preparation for infection

E. coli UTI89 and *S. saprophyticus* 15305 used for infections were inoculated into 10 mL LB directly from -80°C freezer stocks, grown statically overnight at 37°C, reconstituted in fresh LB media with or without ciprofloxacin and grown for another four hours at 37°C. These cultures were then centrifuged for 10 minutes at 3000xG, resuspended in 10 mL of PBS, and diluted to OD_{600} of 1.0 (~2.0 x 10^8 cfu/mL). 50 µL of this suspension was used to inoculate the bladders of mice via transurethral catheterization.

Syringes were prepared for infection by applying segments of polyethylene tubing to the end of a sterile 30-G hypodermic needle until fully covered. The ethylene tubing was cut such that one needle-length was still present after. Enough catheterized needles would be prepared such that one catheter was made available per experimental group. Prepared catheters were exposed to UV radiation for at least 30 minutes for sterilization. If not used immediately, sterile catheters were housed in sterile petri dishes until needed.

2.13.2 Murine sedation, infection, urine collection, and sacrifice

Prior to infection, C3H/HeN mice were exposed to 4% isoflurane for induction of anesthesia, and subsequently 2.5%-3% isoflurane for maintenance of the anaesthetic state with an oxygen flow rate of 0.5 L/minute. However, C57BL/6 mice were induced using the above protocol, but maintained at isoflurane levels no higher than 2%. Anaesthetization was accomplished using a mobile respirator unit containing an E-cylinder oxygen tank, gas regulator, and anesthetic vaporizer with reservoir and waste anesthetic gas scavenging containers. Five mice could be sedated at any one time using this set-up. Mice were constantly monitored for breathing rate to avoid death by asphyxiation. If breathing rate dropped to more than four seconds between breaths mice were removed from anesthetization. Once a breathing rate of ~1 breath per second was achieved, individual mice were removed from the induction chamber and placed flat on their back under an anesthetic nose cone to maintain sedation. Anesthetization was adjusted accordingly if mice showed signs of waking up (for example, rapid breathing and limb movement). Urine was manually expressed by gently massaging and pushing on the bladder. Once urine had been voided, a lubricated, catheterized inoculating syringe containing the bacterial inoculum was fully inserted into the urethra without resistance. Once the catheter was inserted, 50 µL of the bacterial inoculum (~1-2 x 10^7 cfu) was delivered into the bladder at a rate of ~10 µL/second. Following inoculation mice were transferred back into the cage and awoken.

Urine was collected prior to infection, in addition to during the course of infection until sacrifice. This was done by applying suprapubic pressure with proper hand-held restraint to murine bladders and collecting the urine stream in sterile, 1.5 mL eppendorf tubes. At least 20 µL of urine was collected from each mouse. Urine bacterial load (titer) was determined via serial dilution in PBS and spot plating (10 µL per drop, 50 µL total per plate, per sample).

Bladders and kidneys were harvested from mice in order to permit determination of bacterial load in these organs. Prior to sacrifice, mice were transferred glass jars containing cotton balls soaked in isoflurane. Up to five mice were placed into the jar and maintained under anesthetic until agonal breathing was observed or breathing ceased in

all animals. At this time, all animals were removed from the jar, placed stomach down, and sacrificed via cervical dislocation. Sacrificed mice were then placed on their backs, and abdominal regions sprayed thoroughly with 95% ethanol to sterilize the site of incision. The lower abdomen was cut open using surgical scissors, and manipulated using sterile forceps. Bladders and kidneys were excised and placed in either 1 mL or 800 µL of sterile PBS respectively for future homogenization and subsequent bacterial enumeration. Tissues were mechanically homogenized to liberate bacteria, with homogenates maintained on ice until dilution plated (10 µL per drop, 50 µL total per plate, per sample).

2.13.3 *Ex vivo* gentamicin protection assay

S. saprophyticus 15305 were reconstituted 1000-fold from overnight (24 hour) cultures into fresh LB media containing 1/4MIC of relevant antibiotics or vehicle. Samples were incubated at 37°C for four hours and transurethrally infected into C3H/HeN mice as described. Mice were sacrifice at 3.5 hours post-inoculation (hpi), and bladders were excised, bisected twice, and placed in 0.5 mL of PBS. Bladder segments were washed three times with PBS to remove luminal *S. saprophyticus* 15305, then transferred to microfuge tubes containing 1 mL of PBS and 100 µg/mL gentamicin. Samples were incubated for 90 minutes at 37°C on a rocker. After gentamicin treatment, bladder segments were washed two times and placed in 1 mL fresh PBS and homogenized. Whole bladders were plated (250 µL/plate) and incubated for 48 hours at 37°C. Isolated colonies were confirmed as *S. saprophyticus* by spraying plates with a combination solution of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Thermo Scientific) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Thermo Scientific). As *S. saprophyticus* 15305 contains endogenous β-galactosidase, conversion of colonies from white to blue ensured correct identification.

2.13.4 IBC visualization using light-microscopy

E. coli UTI89 were reconstituted 1000-fold from overnight (24 hour) cultures into fresh LB media containing 1/4MIC of relevant antibiotics or vehicle. Samples were incubated at 37°C for 4 hours and transurethrally infected into C3H/HeN mice as described. The animals were sacrificed 6 hpi, and bladders were excised, bisected longitudinally, and placed onto a silicone bladder pinning pad. Silicone pads were prepared using silicone elastomer (SYLGARD 184; Dow Corning, Columbia City, IN) poured into a 6-well plate to about half the well height as per the manufacturer's instructions. Bladders were stretched, flattened and pinned in place to the silicone pad luminal side up. They were then fixed with 3% paraformaldehyde (Sigma), washed thrice with 2 mM $MgCl_2$ (Sigma), 0.01% sodium deoxycholate (Sigma), and 0.02% Nonidet P-40 (Roche, Mississauga, ON) in PBS. Staining was performed using 25 mg/mL X-gal (Sigma), and a solution containing 1 mM potassium ferrocyanide and 1 mM potassium ferricyanide (Sigma). After an incubation period of 16 hours at 30°C, bladders were visualized under an Olympus SZX12 dissecting microscope (Olympus America, Center Valley, PA), and appear as punctate blue stains.

2.13.5 IBC visualization and volumetric analysis using confocal microscopy

IBCs were also visualized using confocal microscopy to permit a more accurate determination of size and morphology. Infections and bladder fixation were conducted as described, with the exception that *E. coli* UTI89 carrying the green fluorescent protein (GFP)-expressing plasmid pANT4 was utilized. pANT4 is stably harbored during acute infection stages (Justice *et al.*, 2004). Fixed bladders were washed and counterstained for 20 minutes with nuclear ToPro3 (Molecular Probes) stain, and recombinant wheat germ agglutinin (r-WGA) to outline superficial umbrella cells (1:700 dilution for each). Bladders were imaged using a Zeiss LSM 510 Meta Laser Scanning inverted confocal microscope (Thornwood, NY). IBCs were rendered in three-dimensions (3D) via reconstructive Z-stacking of images using Volocity 4 image analysis software (PerkinElmer, Waltham, MA). Volumetric analysis was also conducted using Volocity

software, which calculates GFP volume by counting the number of green pixels within a given area of the region defined as an IBC (Anderson *et al.*, 2010).

2.13.6 Empiric ciprofloxacin therapeutic dose determination for murine UTI

Human dosing parameters were used to determine the empiric ciprofloxacin daily dose in mice (Iravani *et al.*, 1999). Recommended dosing for humans is set at ~10 mg/kg (drug/weight), taken bi-daily for typical therapeutic ciprofloxacin treatment of uncomplicated UTI. The average weight of a C3H/HeN mouse is ~20 g. Therefore, these mice required a total daily dose of ~400 µg ciprofloxacin for UTI treatment. The average water intake for a 20 g mouse was empirically determined to be ~10 mL/day. Thus, the drinking water of mice was supplemented with 40 µg/mL of ciprofloxacin to achieve a daily therapeutic dose over the course of 24 hours.

2.13.7 Sub-therapeutic ciprofloxacin and UPEC urine titer

The effect of sub-therapeutic ciprofloxacin dosing on murine response to UTI was investigated in both previously inoculated, but resolved mice in addition to chronically infected mice. The animals were prepared by inoculating with a dose of 10^8 cfu of *E. coli* UTI89 as described previously, and were left for at least 30 days to either spontaneously resolve infection, or develop chronic cystitis, defined as urine titers of $>10^4$ cfu/mL for ≥ 14 dpi (Hannan *et al.*, 2010). The mice that resolved ($<10^4$ cfu/mL ≥ 14 dpi) were separated from those that developed chronic infection into cages containing up to five mice from the same group.

The urine titers of mice were monitored for three days prior to initiation of sub-therapeutic ciprofloxacin supplementation as previously described. This was done to ensure that infections had stabilised in each mouse. Ciprofloxacin was supplemented into the drinking water for *ad libitum* consumption. Water intake was monitored and did not differ between antibiotic-supplemented and control groups indicating ciprofloxacin did

not alter palatability (data not shown). Dosing ranges of <1/25 the empirical therapeutic dose were found to not significantly decrease bacterial urine titer over a three day period (data not shown). Following optimization of dosing parameters, such that UPEC were not negatively influenced by the level of ciprofloxacin present, 1/50th the empirical ciprofloxacin therapeutic dose was supplemented into the drinking water and replaced each day for 3-6 days. The urine titer of each mouse was monitored daily for changes as described. At either three or six days, the dose was further increased to 1/25th the empirical therapeutic dose for another 3-6 day period with urine titers again determined daily. Lastly, the dose was increased further to 1/10th the empirical therapeutic dose for three days to clear infection and ensure spontaneous development of ciprofloxacin resistant mutants did not occur.

2.13.8 Human and murine tissue cytokine quantification

The influence of sub-therapeutic ciprofloxacin dosing on immune activation was assessed during the early stages of infection. Prior to infection, mice were supplemented with 1/50th the empirical ciprofloxacin therapeutic dose for three days. Infections, as described previously, were established by inoculation of 10^7 non-treated *E. coli* UTI89 or control PBS (mock) into mice receiving sub-therapeutic ciprofloxacin or no antibiotic. Infections were allowed to develop for 3.5 hours to permit activation of early immune response elements. At this time, urines were extracted for later analysis, mice sacrificed, and bladders and kidneys excised and snap frozen in liquid nitrogen for future analysis.

The cytokine expression profiles for murine bladders was conducted using a Luminex® bead-based assay. Cytokines were liberated from tissues using homogenization, as previously described, in extraction buffer containing 20 mM Tris-HCl (Sigma; pH 7.5), 150 mM NaCl (Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma), 0.05% Tween-20 (Sigma), and a protease inhibitor cocktail (100-fold dilution; Roche). Conversely, supernatants from *in vitro* human T24 tissue cell line experiments were also extracted and analysed. Extracts were maintained on ice, centrifuged at 14,000 rpm for three minutes, and supernatants removed and stored at -80°C until use. Total protein

levels were assess using a bicinchoninic acid (BCA) kit (Thermo Scientific) as per the manufacturer's instructions and diluted when required.

Levels of cytokines IL-1β, IL-6, keratinocyte chemoattractant (KC - also referred to as IL-8), granulocyte colony-stimulating growth factor (G-CSF), IL-17, IL-10, and tumor necrosis factor alpha (TNFα) were measured using multiplexed immunoassay kits according to manufacturers' instructions (Bio-Rad Laboratories Inc., Hercules, CA). The appropriate mouse or human kits were used for each sample. A Bio-Plex 200 readout system was used (Bio-Rad), which utilizes Luminex® xMAP fluorescent bead-based technology (Luminex Corporation, Austin, TX). Cytokine levels (pg/mL) were automatically calculated from standard curves using Bio-Plex Manager software (v. 4.1.1, Bio-Rad). Standard curves were generated in extraction buffer containing no protease inhibitors.

Enzyme-linked immunosorbent assays (ELISA) were used to determine IL-8 expression levels in human 5637 bladder cells. Infections were carried out as previously described (*In vitro* tissue culture infection protocol), with the exceptions that lipopolysaccharide (LPS; Sigma) was added at 5 µg/mL in place of *S. saprophyticus* 15305 as a cell-free control, and ampicillin and gentamicin were also tested at 1/4MIC levels in addition to ciprofloxacin. At four hours after incubation of the pathogen with the 5637 cells, supernatants were removed, along with bacteria and cellular debris via centrifugation (14,000xG for three minutes), and the resulting extracts placed in the wells of a 96-well plate and frozen at -80°C until needed. Cytokines were quantified using the Human CXCL8/IL-8 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions.

2.13.9 Quantification of urine sediment polymorphonuclear leukocyte infiltration

Urines collected at 3.5 hpi from mice receiving 1/50th the empirical ciprofloxacin therapeutic dose were further analysed for their immune cell content. Urine sediments were obtained by cytocentrifuging 80 µL of a 10-fold (PBS) diluted urine sample onto

poly-L-lysine-coated glass slides using a CytoPro 7620 cytocentrifuge (Wescor, Logan, UT). Samples were briefly heat fixed and stained with filter sterilized Protocol 3 Hema 3 stains (Wright-Giemsa method, Fisher Scientific). Stained urine sediments were examined by light microscopy on an Olympus BX51 light microscope (Olympus America), and the average number of polymorphonuclear leukocytes (PMN) per 400x magnification field (high powered field) calculated from counting at least five fields. A semi-quantitative scoring system was used for analysis and is described in Table 2.2.

2.13.10 Model of ciprofloxacin prophylaxis

In general, the proof-of-principle antibiotic dosing strategies used throughout this work are not reflected in real-world therapeutic scenarios. However, low-dose, extended use prophylactic strategies are often implemented for the management of recurrent UTI. These aim to achieve sub-inhibitory levels throughout the daily dosing period. It is possible that these transient, low-dose periods are sufficient to enhance *E. coli* UTI89 virulence. Therefore, a mock prophylaxis dosing scheme was applied to the current murine infection model. C3H/HeN mice were abandoned for prophylaxis experiments as they have a congenital anatomical defect which promotes vesicoureteral reflux and subsequent kidney infection (Hung *et al.,* 2009; Hopkins *et al.*, 1995). Instead, C57BL/6 mice were utilized to avoid this issue. The model involved a 24 hour infection period as described previously, followed by a three day regimen of normal co-trimoxazole therapy as described by Schilling *et al.* (2002) (270 μg/mL replaced daily; Qualitest Pharmaceuticals, Huntsvill, AL). Together these periods mimic an infection event, symptom development, and subsequent initiation of antibiotic therapy. Following therapy cessation, mice were provided a seven day rest period prior to initiation of ciprofloxacin prophylaxis. They received either a typical prophylactic dose of ciprofloxacin (1/4th the empirical therapeutic dose) or vehicle (dH_20) daily via oral gavage for seven days. Oral dosing was conducted by injecting 100 μL of 1 μg/mL ciprofloxacin directly into the stomachs of mice. Following prophylactic therapy, animals were sacrificed, and kidneys and bladders excised for bacterial titer, as described earlier. In some cases, murine bladders were gentamicin treated for determination of the intracellular population. Urine

was taken at least every two days throughout the course of the experiment to allow tracking of infection.

Table 2.2 PMN Categorical Scoring Scheme

Total PMNs[a]	**Score**
0-4	0
5-24	1
25-49	2
50-99	3
100+	4

[a] Per 5x high-powered fields of views [hpf (40x objective)].

2.14 TYPE I AND TYPE II PERSISTER CELL ASSAYS

Type I persisters were isolated by directly applying lethal concentrations of ciprofloxacin (5 µg/mL), ampicillin (100 µg/mL), or gentamicin (10 µg/mL) to stationary phase (24 hour) cultures of *E. coli* UTI89, PAS0209, PAS0211, CFT073 or *S. saprophyticus* 15305 for three hours and enumerating the surviving fraction before and after treatment (Keren *et al.*, 2004). Conversely, overnight cultures were first seeded 1000-fold into fresh LB and then grown for ~3 or ~7 hours (for UPEC and *S. saprophyticus* respectively) to deplete the Type I persister population for Type II persister assays (until early-exponential phase; turbidity of 0.5-0.6 for *S. saprophyticus* and *E. coli* at 600 nm). A subsequent 1000-fold seed in fresh LB with 1/4MIC antibiotics lasting ~3-7 hours (turbidity of 0.5-0.6 at 600 nm) was performed to enrich the Type II persister fraction. Following this incubation period, cells were washed and percent survival was determined through the enumeration of viable cells prior to and following treatment with lethal antibiotics. Colonies were isolated by serially diluting cultures in PBS, spot-plating 10 µL drops on LB agar, and counting the resulting colonies. In cases where viability fell below 1000 cfu/mL, 10 or 100 µL drops would be applied directly from cultures reconstituted in PBS and spread over the entire surface of an LB plate. Survival due to phenotypic tolerance and not spontaneous resistance was determined by sub-culturing the surviving fraction in fresh LB and determining the MIC.

2.14.1 Persister resuscitation assays

The kinetics of persister resuscitation were determined for both *E. coli* CFT073 and *S. saprophyticus* 15305 following Type II persister assays. After treating cultures with relevant antibiotics at lethal levels, cultures were resuspended in fresh LB, serially diluted into the wells of a 96-well plate containing LB, sealed and grown for 21 days at 37°C. Wells were marked daily as positive when turbidity was observed.

2.15 AUTOAGGREGATION ASSAY

It is well established that organisms in the confinement of adherent high-density biofilms are much more refractory to antibiotic therapy then their planktonic counterparts. Importantly, this trait is not due to the inability of antibiotics to penetrate the extracellular matrix, as most agents readily diffuse to the cores of such films (Singh *et al.*, 2010). Therefore, persister cells have been labelled as the biofilm constituents which confer high-grade MDT. In addition to surface-dependent biofilm formation, rapid planktonic-phase aggregation has a positive influence on antibiotic tolerance and host immune cell evasion while enhancing adherence properties (Boll *et al.*, 2013; Haaber *et al.*, 2012; McNabe *et al.*, 2011). However, it is unknown whether high-density growth is a contributor or side-effect of high frequency persisters within biofilms. A contributing role seems plausible as persisters comprise a significantly larger fraction of the overall population as cultures reach stationary phase.

Autoaggregation was quantified in a number of ways. First, antibiotic effects on this process were considered. The protocols used have been described, and involved quantification via the microscope adherence assay protocol for *S. saprophyticus* 15305 aggregation, and negative staining and analysis by transmission electron microscopy protocol for *E. coli* UTI89 aggregation. In both cases, aggregation was considered as the number of associated organisms per individual aggregate. Visualization of individual *E. coli* UTI89 in aggregates via light microscopy was not reliable, necessitating the TEM approach which was used instead.

A third protocol was utilized to compare levels of stationary-phase, type 1 fimbriae-dependent aggregation in the absence of antibiotics. This was accomplished using a sedimentation assay, which measures the rate of settling over time as a function of aggregate size. Type 1 fimbriae deficient *E. coli* UTI89 mutants Δ*fimH* (pili expressed without adhesin) and Δ*fimS* (pili not expressed - bald phenotype) were utilized. Organisms were grown to stationary phase as previously described. At this point, cells were harvested via gentle centrifugation at 4500xG for 10 minutes. Pellets were resuspended in PBS to an OD_{600} of 1.0. Cell suspensions were then vortexed for ~10 seconds and incubated at room temperature for five hours to permit sedimentation. Every

hour, 0.1 mL of the upper suspension was gently removed and transferred to 0.9 mL of PBS and the OD_{600} measured. The autoaggregation determined was expressed as 1-(A_t/A_0)x100, where A_t represents the absorbance at time t = 5 hours and A_0 the absorbance at t = 0.

2.16 TRANSCRIPTOMIC ANALYSES

2.16.1 Bacterial preparation, RNA extraction and enrichment protocols

RNA sequencing was conducted in order to gain a better understanding of how transcription is modulated in response to sub-inhibitory antibiotics, and to complement the phenotypic changes already characterized. Both *S. saprophyticus* 15305 and *E. coli* UTI89 were grown in the presence or absence 1/4MIC ciprofloxacin until an OD_{600} of 1.0 was achieved. An additional treatment group included the application of 1/4MIC ciprofloxacin at OD_{600} of 1.0 for 30 minutes to determine the early effects of sub-inhibitory antibiotic on transcript expression. After these incubation periods, RNA protect was directly added at a ratio of 2:1 RNA protect:sample for 10 minutes. Solutions were centrifuged at 5,000xG for five minutes and pellets frozen at -80°C until required for RNA extraction.

S. saprophyticus 15305 pellets were resuspended in 1 mL TE buffer containing 10 mM Tris-HCl, 1 mM EDTA, 20 mg/mL lysozyme (Sigma), and 50 U/mL lysostaphin (Sigma) at 37°C for 30 minutes, while *E. coli* UTI89 was incubated in the same buffer without lysostaphin for the same period. Samples were then centrifuged at 5,000xG for five minutes, supernatants discarded, and pellets resuspended in 1 mL TRIzol (Ambion, Burlington, ON) reagent by vortexing followed by a 10 minute incubation period at room temperature. 200 µL of chloroform was added to each sample then vortexed for 15 seconds and incubated at room temperature for 10 minutes followed by centrifugation at 16,000xG for 15 minutes. 500 µL of the upper aqueous phase was transferred to an RNase-free tube and 500 µL of 100% ethanol was added then vortexed. Samples were

then run through a PureLink RNA Mini Kit (Ambion) to purify the extracted RNA as per the manufacturer's instructions. In all instances, RNA was eluted in 30 µL volumes using RNase-free water. RNA quality and purity was determined at Robarts Research Institute, London Regional Genomics Centre using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) and the RNA 6000 Pico Kit (Caliper Life Sciences, Mountain View, CA). Only samples containing high-quality RNA [RNA integrity numbers (RINs) $\geq$ 9.0] were used for further analysis and sequencing. mRNA was enriched prior to sequencing using the MICROB*Express* kit (Ambion). A single enrichment run was sufficient to deplete 16S and 23S ribosomal RNA from the *S. saprophyticus* 15305 samples; however, *E. coli* UTI89 samples required two depletions prior to sequencing. The purity and quantity of the RNA was assessed prior to enrichment using a Qubit 2.0 Fluorometer (Life Technologies, Burlington, ON) and RNA BR Assay (Life Technologies). RNA was added to the MICROB*Express* kit at a concentration of 0.6 µg/mL in 15 µL total volume and extracted in RNase-free water following ethanol precipitation. Effective ribosomal RNA depletion was confirmed using a bioanalyzer.

2.16.2 RNA library preparation and sequencing

Purified and depleted RNA was barcoded using ScriptSeq Index PCR Primers 1-19 (Epicenter Biotechnologies, Madison, WI) and pooled into a library at Robarts Research Institute, London Regional Genomics Centre using the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre). Briefly, samples were fragmented, cDNA was synthesized, tagged, cleaned-up and subjected to PCR with barcoded reverse primers to permit equimolar pooling. In total, 50 ng of RNA was used for each library preparation. The quality and quantity of the library preparation was confirmed via bioanalyzer (Agilent High Sensitivity DNA chip) and qPCR Library Quant Kits (KAPA Biosystems, Wilmington, MA), with primers specific to end library fragments. Samples were clustered via cBOT (Illumina, Toronto, ON) and sequenced using the HiSeq 2000 platform (Illumina) at The Biodiversity Research Centre, NGS Facility (University of

British Columbia). Samples were run on two lanes at 12 pM using 100 bp, paired-end reads as per Illumina specifications and protocols.

2.16.3 Reference sequence library and mapping

E. coli UTI89 [accession NC_007946.1 (Chen *et al*., 2006)] and *S. saprophyticus* 15305 [accession NC_007350.1 (Kuroda *et al*., 2005)] were included for mapping. These datasets included the predicted coding sequences extracted from the complete genomes and plasmids available from the National Center for Biotechnology Information (NCBI) database. Reads uniquely mapped to coding sequences using BOWTIE 2.2.1 (Langmead and Salzberg, 2012) were included in the differential expression analysis (all other unmapped reads were discarded). Reads were trimmed by 20 nt from the 5' end and 40 nt from the 3' end, and a maximum of two mismatches were allowed. Reads with equal best hits were mapped at random to one of the locations. Amino acid translations of predicted coding sequences were compared using SEED Subsystems (Overbeek *et al*., 2005) and assigned by blastp with an e-value cutoff of 1e-3. The KEGG Automatic Annotation Server (KAAS) (Moriya *et al*., 2007) was used for annotation of enzyme functions (KOs).

2.17 STATISTICAL ANALYSES

Statistical analyses were conducted using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Significance was determined using one-way ANOVA and Dunnett's multiple comparison test or Bonferroni's multiple comparison test, Mann-Whitney test with Gaussian approximation, Logrank test, and two-tailed or unpaired t-test. Each test used for respective experiments is detailed in its figure legend. Statistical analyses for the differential expression of reads generated using RNA sequencing were conducted using the ALDEx R package version 2.0.6 (Fernandes *et al*., 2013; Fernandes *et al*., 2014 - awaiting publication). A $\log_2$ median effect size of at least 1.5 was required for genes to be considered differentially expressed. In addition, a less

than 1% overlap in the distributions between the two conditions was permitted for inclusion as described elsewhere (Fernandes *et al.*, 2013; Macklaim *et al*., 2013).

CHAPTER THREE

RESULTS

3 RESULTS

3.1 EXAMINING THE EFFECTS OF SUB-INHIBITORY ANTIBIOTICS ON UROPATHOGEN VIRULENCE

The notion that sub-inhibitory antibiotics demonstrate signalling capacity in prokaryotes is of clinical concern and certainly warrants investigation. This is especially true in situations where antibiotics are applied at low-doses for prolonged periods of time such as during prophylactic management of recurrent UTI. Thus far, the majority of studies exploring phenomenon have utilized transcriptomics and reporter strains to illustrate global changes in gene expression. However, there is a severe deficit in our understanding of what exactly these changes accomplish on the phenotypic level, and how they influence virulence and pathogenesis. An aim of the present study was to investigate the capacity of sub-inhibitory antibiotics to influence various aspects of uropathogen virulence *in vitro*.

3.1.1 Characterizing uropathogen strain sensitivity to various antibiotics

Antibiotic dosing strategies were optimized against all uropathogens utilized before undertaking this work. Initial susceptibility screening was carried out using the Kirby-Bauer method, which indicated that all strains demonstrated clinical susceptibility (Table 3.1). Minimal inhibitory concentration (MIC) cut-offs were generated using the broth microdilution procedure as outlined by the Clinical and Laboratory Standards Institute (CLSI; 2007) (Table 3.1). *S. saprophyticus* strains were comparatively more tolerant of ciprofloxacin than *E. coli* isolates. Conversely, *E. coli* strains were much less sensitive to ampicillin and gentamicin challenge.

Table 3.1 Summary of strain MICs and antibiotic susceptibilities

	MIC (µg/mL)			ZOI (mm [susceptibility])[a]		
Strain (type)	CIP	AMP	GEN	CIP	AMP	GEN
S. saprophyticus strains						
15305	0.25	0.125	0.0625	26.5 (S)	28.25 (S)	20 (S)
15305:C1	0.25	0.125	0.0625	26.5 (S)	28.25 (S)	20 (S)
7108	0.25	0.25	0.0625	26 (S)	28 (S)	21 (S)
E. coli strains						
CFT073	0.0156	3	1	25.5 (S)	17 (S)	17 (S)
TOP277 (SSR)	0.0125	6	0.75	22.75 (S)	19.75 (S)	16 (S)
TOP263 (SSR)	0.0166	8	0.5625	23.5 (S)	16.25 (S)	16 (S)
TOP379 (SSR)	0.0208	10	0.6875	23.25 (S)	14 (I)	15 (S)
TOP344 (SSR)	0.0229	10	0.875	21.75 (S)	14 (I)	15 (S)
TOP345 (SSR)	0.0146	8.5	0.875	26 (S)	19.75 (S)	16.25 (S)
PUTS277 (AI)	0.0208	9	0.3125	26.25 (S)	17.5 (S)	17.25 (S)
PUTS278 (AI)	0.0166	6	0.375	25 (S)	17.25 (S)	17.75 (S)
PUTS1127 (AI)	0.0166	9.5	1	28 (S)	17.75 (S)	16 (S)
PUTS1236 (AI)	0.0166	9	0.75	23.5 (S)	17.25 (S)	16.5 (S)
UTI89	0.0188	9	1.25	26.5 (S)	16.75 (S)	15.75 (S)
UTI89 (Δ*recA*)	0.0009	8	1	43.75 (S)	25.75 (S)	25.25 (S)
UTI89 ($lexA_{T355G}$)	0.0039	8	1.25	35.5 (S)	22.75 (S)	21 (S)

[a] ZOI, zone of inhibition; S, susceptible; I, intermediately susceptible; R, resistant (per CLSI guidelines).

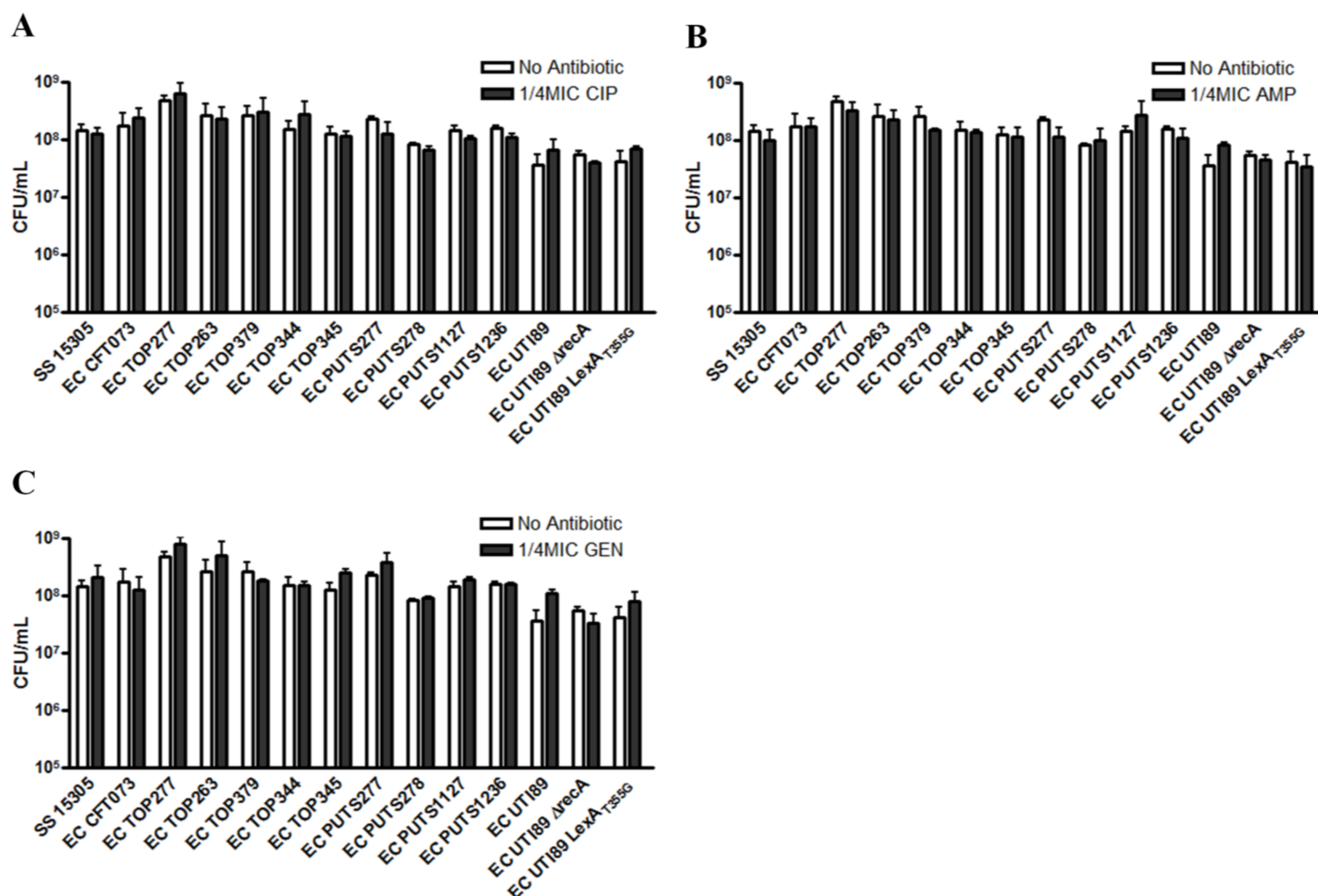
Optimal sub-inhibitory antibiotic dosing strategies were evaluated to determine concentrations which confer phenotypic effects without perturbing growth. Although a range of concentrations were considered, 1/4MIC levels were the maximal dose that organisms could tolerate without loss of fecundity (Figure 3.1). Therefore, this dose was used throughout unless stated otherwise.

3.1.2 Evaluating the ability of sub-inhibitory antibiotics to induce adherence of *S. saprophyticus* to abiotic surfaces

Colonization of surfaces represents one of the first critical steps in bacterial infection. Therefore, the influence of antibiotic stress on this process was the first trait to be evaluated in *S. saprophyticus* 15305. Initially, experiments were carried out using human pooled urine (HPU) as a growth medium. Surfaces which come into contact with urine are immediately coated with a conditioning film which is composed of various urine metabolites and host proteins such as immunoglobulins, Tamm-Horsfall protein, histones, and nuclear DNA-condensing proteins (Canales *et al.*, 2009), and are important in surface-pathogen adherence potential. To achieve a basic understanding of antibiotic influence on adherence, trials were first carried out on glass microscope slide surfaces to permit rapid visualization of experimental outcomes. Early-exponential phase *S. saprophyticus* 15305 cultures were exposed to 1/4MIC antibiotics or control for one hour prior to incubation on slide surfaces. This time point was selected to demonstrate the rapid influence of antibiotics on colonization processes. Following a brief wash period to remove non-irreversibly attached organisms, slides were stained with crystal violet and imaged using a light microscope. Representative light-micrographs of all samples revealed the presence of adherent *S. saprophyticus* as punctate purple spots, with antibiotic-primed groups demonstrating visibly larger populations (Figure 3.2A). Enumeration of randomly selected fields of view (FOV) for each treatment group, and comparison with HPU control populations demonstrated that antibiotics are effective in increasing the relative adherence in primed pathogens (Figure 3.2B). Specifically, adherence was significantly increased with 1/4MIC ciprofloxacin and ampicillin treatments (37%; $P < 0.05$), and 1/4MIC gentamicin treatments (50%; $P < 0.01$).

Figure 3.1. Comparison of cfu/mL between subinhibitory antibiotic pre-treated and untreated uropathogens immediately prior to lethal antibiotic challenge. Negligible impact of antibiotic pre-treatment on uropathogen growth receiving either 1/4MIC ciprofloxacin (A), 1/4MIC ampicillin (B) or 1/4MIC gentamicin (C) for three hours is shown. Means are from at least three independent experiments. Significance determined using paired t-test for each pair-wise combination (all combinations are non-significant; $P > 0.05$).

Figure 3.1

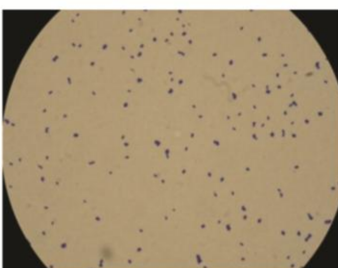


Demonstrating that antibiotics can modulate bacterial adherence capacity to hydrophilic glass warranted further exploration of this phenotypic change using hydrophobic surfaces. Foley urethral catheters and InLay Optima® ureteral stents were selected as target surfaces both for their hydrophobic properties and application as medical devices for the management of urinary obstruction. These devices are widely used in urology, are readily colonized by uropathogens, and are often placed in conjunction with single-dose prophylactic antibiotics as per AUA best practice policies (Wolf *et al*., 2008). *S. saprophyticus* 15305 was incubated in either the presence or absence of 1/4MIC antibiotic and 1 cm sections of either stent or catheter in LB broth (HPU was discontinued for adherence assays due to the reproducibility issues associated with batch variations). Antibiotic-primed organisms demonstrated an increased capacity to adhere to both stent and catheter surfaces (Figure 3.3), as was found on hydrophilic glass slides. *S. saprophyticus* 15305 adhered more readily to catheters, with antibiotic-primed groups demonstrating significantly larger adherent populations. Adherence increased by 5.9, 6.1, and 6.2-fold on urethral catheters in the presence of 1/4MIC ciprofloxacin, ampicillin, and gentamicin respectively ($P < 0.05$), and by 2.6, 2.6, and 2.7-fold on ureteral stents ($P < 0.05$; $P < 0.01$).

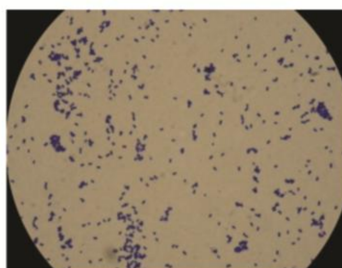
Figure 3.2. Representative images of *S. saprophyticus* 15305 adhering to glass microscope slide surfaces in the presence or absence of 1/4MIC of various antibiotics (A) and relative adherence (B). Attachment percent indicated as a function of the human pooled urine (HPU) control. Means from at least three independent experiments are shown with significance. Significance was determined using one-way ANOVA and Dunnett's multiple comparison test (*, $P < 0.05$; **, $P < 0.01$).

Figure 3.2

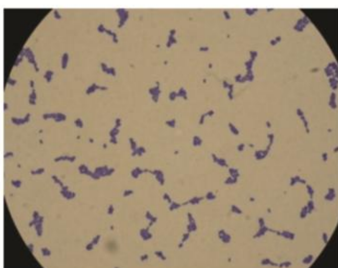
A



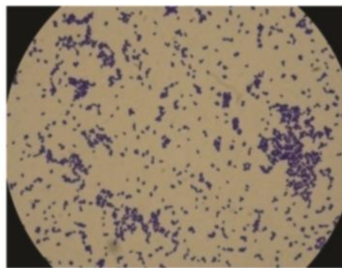
Human Pooled Urine (HPU)



Ampicillin



Ciprofloxacin



Gentamicin

B

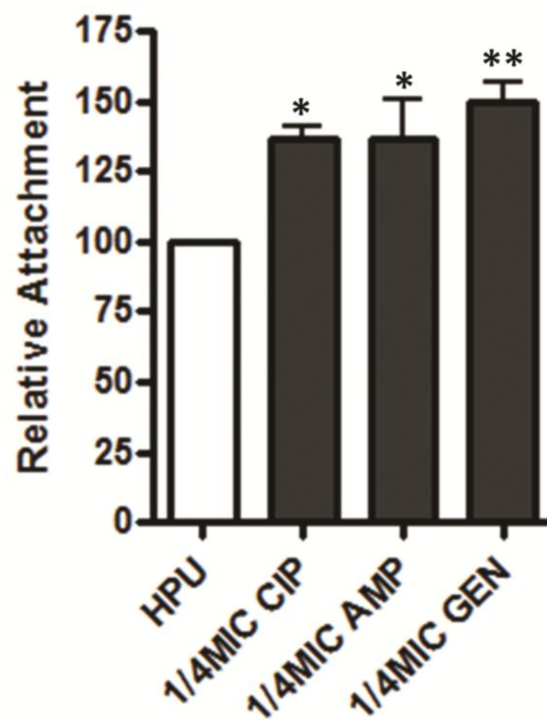
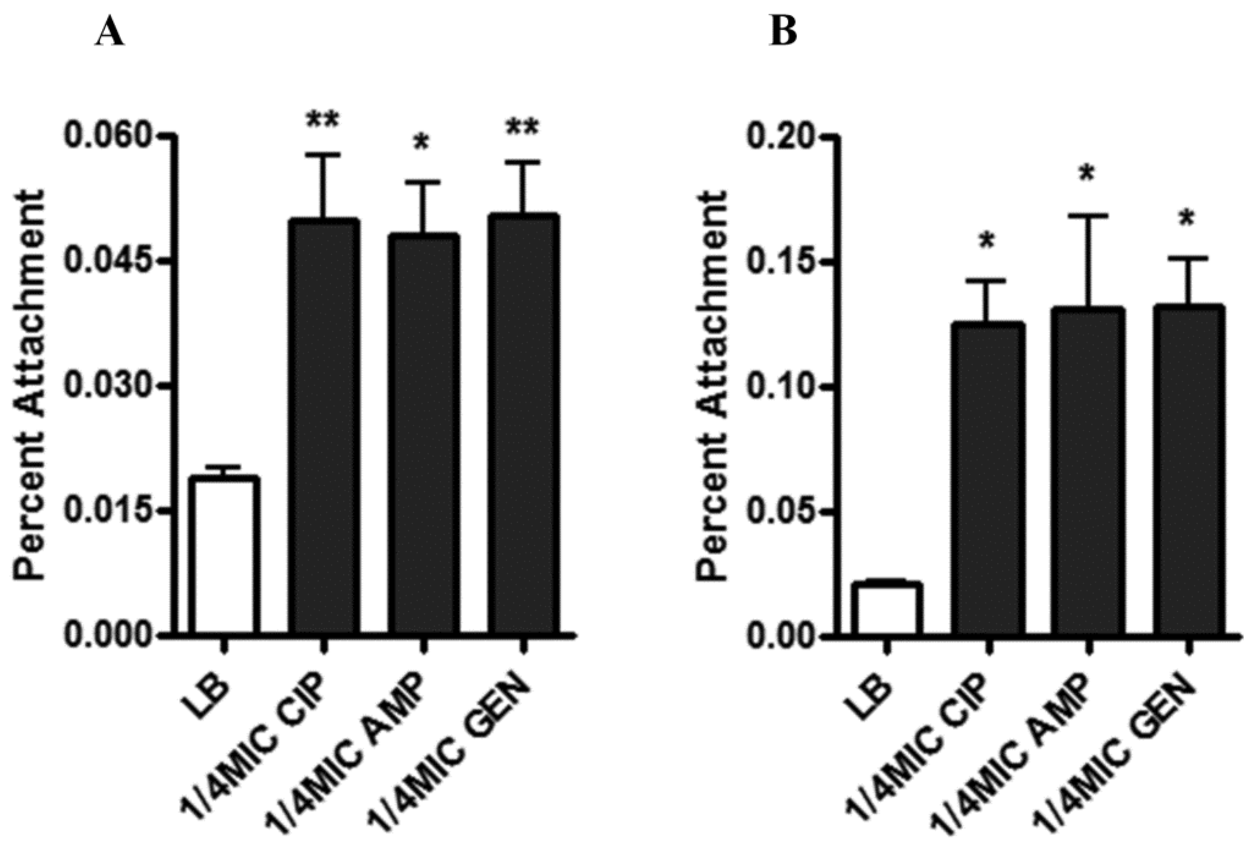


Figure 3.3. Percent adherent *S. saprophyticus* 15305 of the total population (adherent and non-adherent) to InLay Optima ureteral stent (A) or Foley urethral catheter (B) segments in the presence or absence of 1/4MIC of various antibiotics. Means from at least three independent experiments are shown with significance. Significance was determined using one-way ANOVA and Dunnett's multiple comparison test (*, $P < 0.05$; **, $P < 0.01$).

Figure 3.3



3.1.3 Evaluating the ability of sub-inhibitory antibiotics to modulate biofilm formation in *S. saprophyticus* and *E. coli*

Adhesion of uropathogens to surfaces represents the first steps in establishing acute colonization during UTI. Since sub-inhibitory antibiotics were effective in increasing initial bacterial adherence, it was postulated that they would also be able to enhance their biofilm-forming capacity. This was explored by incubating *S. saprophyticus* 15305 in 96-well plates for 24 and 48 hours in the presence or absence of sub-inhibitory antibiotic concentrations. Ciprofloxacin, ampicillin, and gentamicin increased maximal biofilm mass by 38.5%, 34%, and 27.8% respectively compared to untreated controls ($P < 0.05$; $P < 0.01$) (Figure 3.4A). Although antibiotics also were able to enhance biofilm mass after 48 hours, the effect was comparatively much less pronounced compared to 24 hours (Figure 3.4B), with, ciprofloxacin, ampicillin, and gentamicin increasing maximal biofilm amount by only 13.6%, 11.7%, and 12.8% respectively ($P < 0.05$). This plateau in biofilm mass is likely due to saturation effects as *S. saprophyticus* 15305 biofilms approach the maximal capacity supported within the well of the plate.

Compared to *S. saprophyticus*, *E. coli* is more commonly associated with biofilm-related UTI, especially when indwelling urinary devices are present. Previous work has demonstrated this organism can increase biofilm mass in response to aminoglycoside antibiotics (Hoffman *et al.*, 2005). The biofilm forming capacity of *E. coli* UTI89 was also determined following sub-inhibitory antibiotic exposure. The experiments were carried out as described for *S. saprophyticus* 15305, with the exception that only a 24 hour time-point was considered. Sub-inhibitory ciprofloxacin, ampicillin, and gentamicin increased maximal biofilm amounts by 57.7%, 60.0%, and 57.9% respectively ($P < 0.05$; $P < 0.01$) (Figure 3.5).

Figure 3.4. *S. saprophyticus* 15305 biofilm formation in the presence of various antibiotic concentrations at 24 (A) and 48 (B) hours. Means from at least three independent experiments are shown with significance. Significance was determined using one-way ANOVA and Dunnett's multiple comparison test (*, $P < 0.05$; **, $P < 0.01$).

Figure 3.4

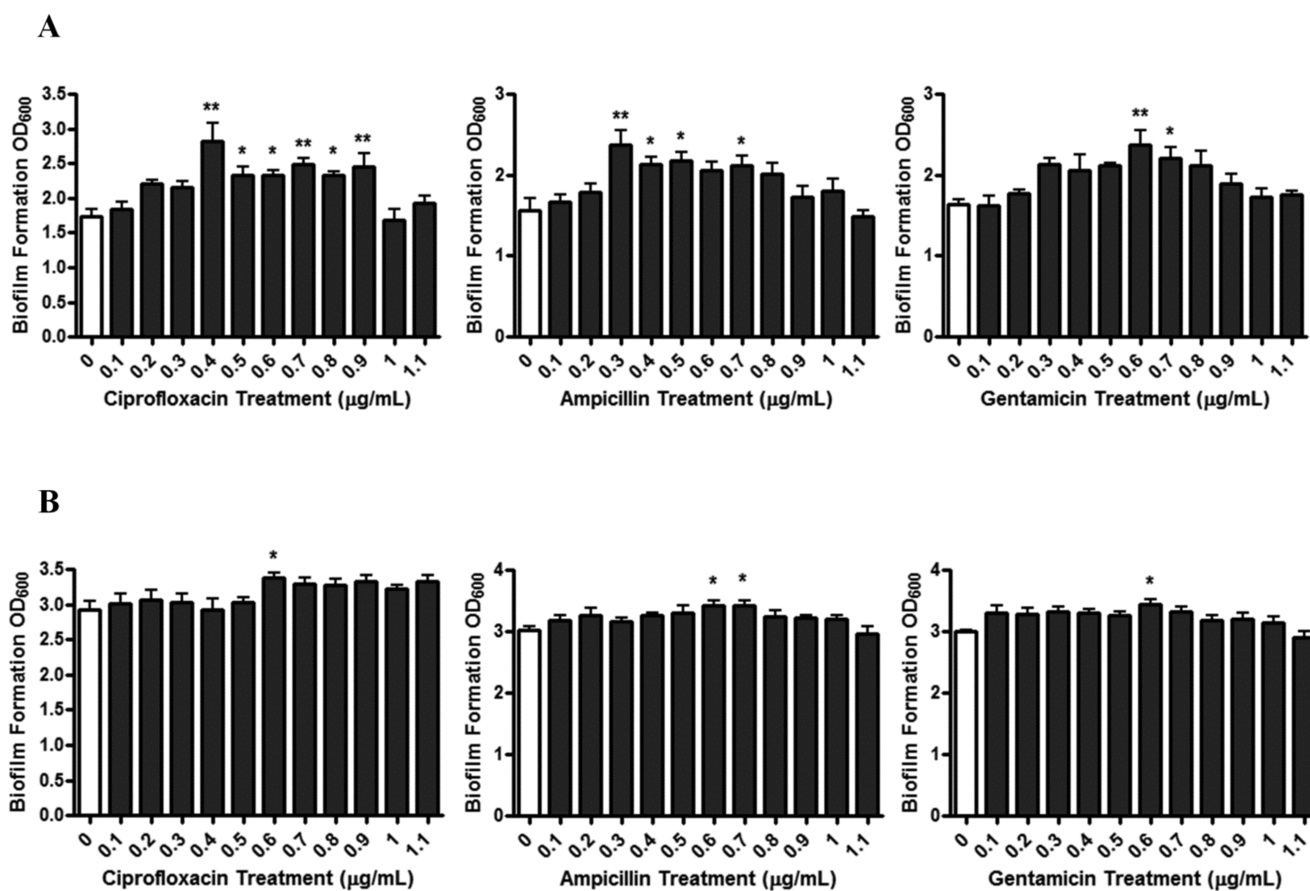
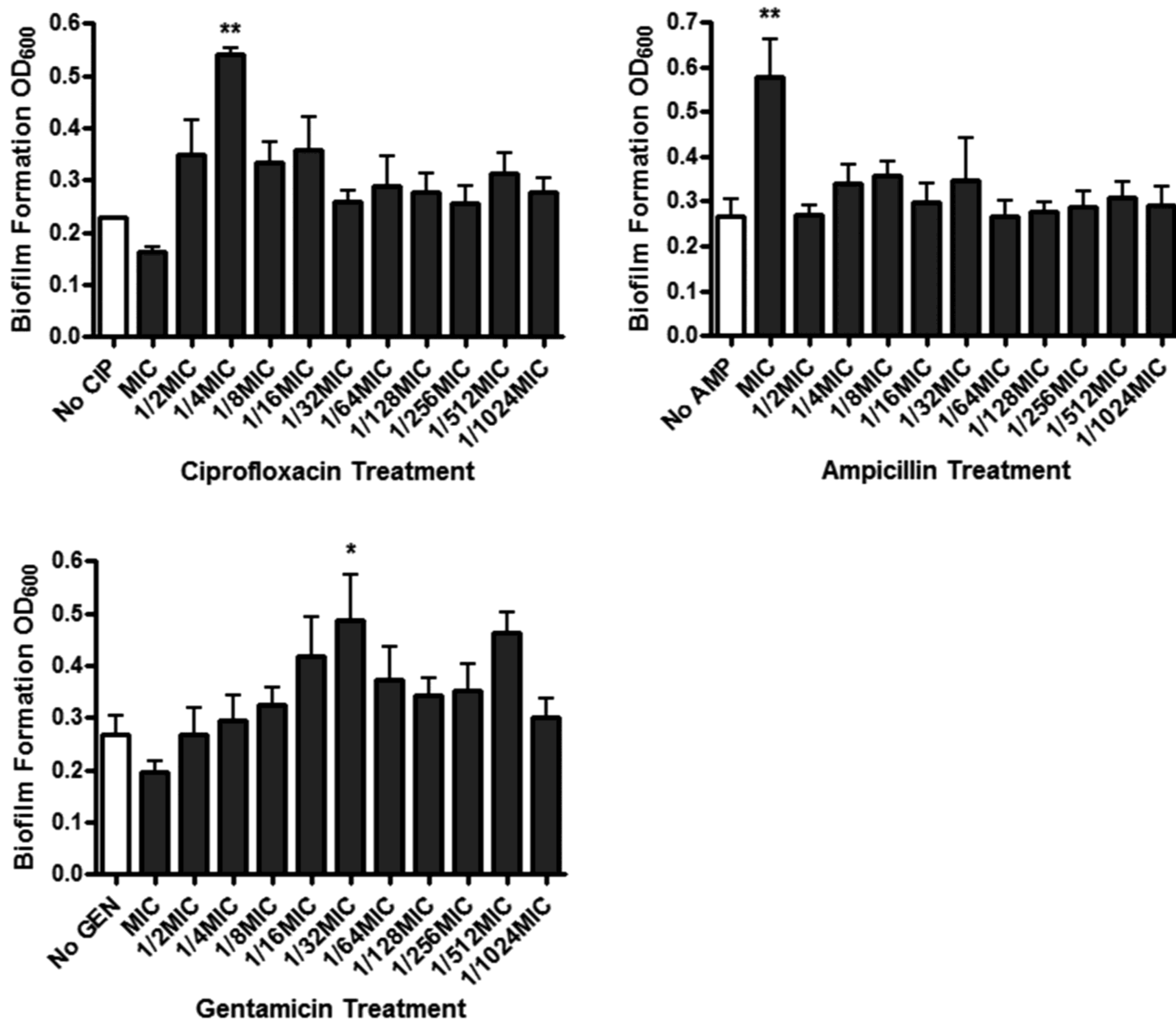


Figure 3.5. Biofilm formation of wild-type *E. coli* UTI89 after 24 hours in the presence of various antibiotic concentrations. Means from at least three independent experiments are shown with significance. Significance was determined using one-way ANOVA and Dunnett's multiple comparison test (*, $P < 0.05$; **, $P < 0.01$).

Figure 3.5



3.1.4 Assessing the contribution of the SOS response to antibiotic-dependent biofilm formation

It is well known that the SOS response is activated following dosing with various antibiotics (Miller *et al.*, 2004; Mesak *et al.*, 2008). However, it has more recently been implicated in the regulation of various aspects of pathogenicity (Bisognano *et al.*, 2000; Kelley *et al.*, 2006). Here, the involvement of the SOS response to antibiotic-dependent biofilm formation is considered. SOS-deficient *E. coli* UTI89 strains PAS0209 and PAS0211 demonstrated a modest suppression in biofilm formation, amassing only 69.6% and 74.3% of the wild-type *E. coli* UTI89 levels respectively ($P < 0.05$) (Figure 3.6A). Next, the contribution of SOS activation during antibiotic-dependent biofilm formation was assessed. *E. coli* PAS0209 biofilm formation was not significantly influenced by ciprofloxacin and gentamicin antibiotics ($P > 0.05$) (Figure 3.6B, D). However, similar to wild-type *E. coli* UTI89, PAS0209 formed more robust biofilms following ampicillin treatment (Figure 3.6C), increasing mass by 40.0% compared to untreated controls ($P < 0.05$). This result was also observed in *E. coli* PAS0211, with ampicillin treatment increasing biofilm mass by 36.6% ($P < 0.05$) (Figure 3.6F). Although no appreciable changes were noted following ciprofloxacin treatment, gentamicin was able to increase biofilm mass by 29.0% ($P < 0.05$) (Figure 3.6E, G).

3.1.5 Antibiotics induce bacterial aggregation

Initial light micrograph images appeared to exhibit *S. saprophyticus* 15305 cultures that would aggregate in the presence of sub-inhibitory antibiotics (Figure 3.2A). In order to investigate whether this process is an important contributor to therapy failure, qualitative aggregation in the presence or absence of sub-inhibitory ciprofloxacin was determined via direct visualization using scanning electron microscopy (SEM) for *S. saprophyticus* 15305 (Figure 3.7A, B) and transmission electron microscopy (TEM) for *E. coli* UTI89 (Figure 3.7C, D). Representative images of control (Figure 3.7A, C) and 1/4MIC ciprofloxacin treated (Figure 3.7B, D) organisms reveal extensive aggregation when ciprofloxacin is present.

Figure 3.6. Biofilm formation of *E. coli* UTI89 and SOS-deficient strains after 24 hours (A). Biofilm formation of *E. coli* SOS-deficient strains *ΔrecA* (B-D) and $lexA_{T355G}$ (E-G) after 24 hours in the presence of various antibiotics at subinhibitory concentrations. Means from at least three independent experiments are shown with significance. Significance was determined using one-way ANOVA and Dunnett's multiple comparison test (*, $P < 0.05$; **, $P < 0.01$).

Figure 3.6

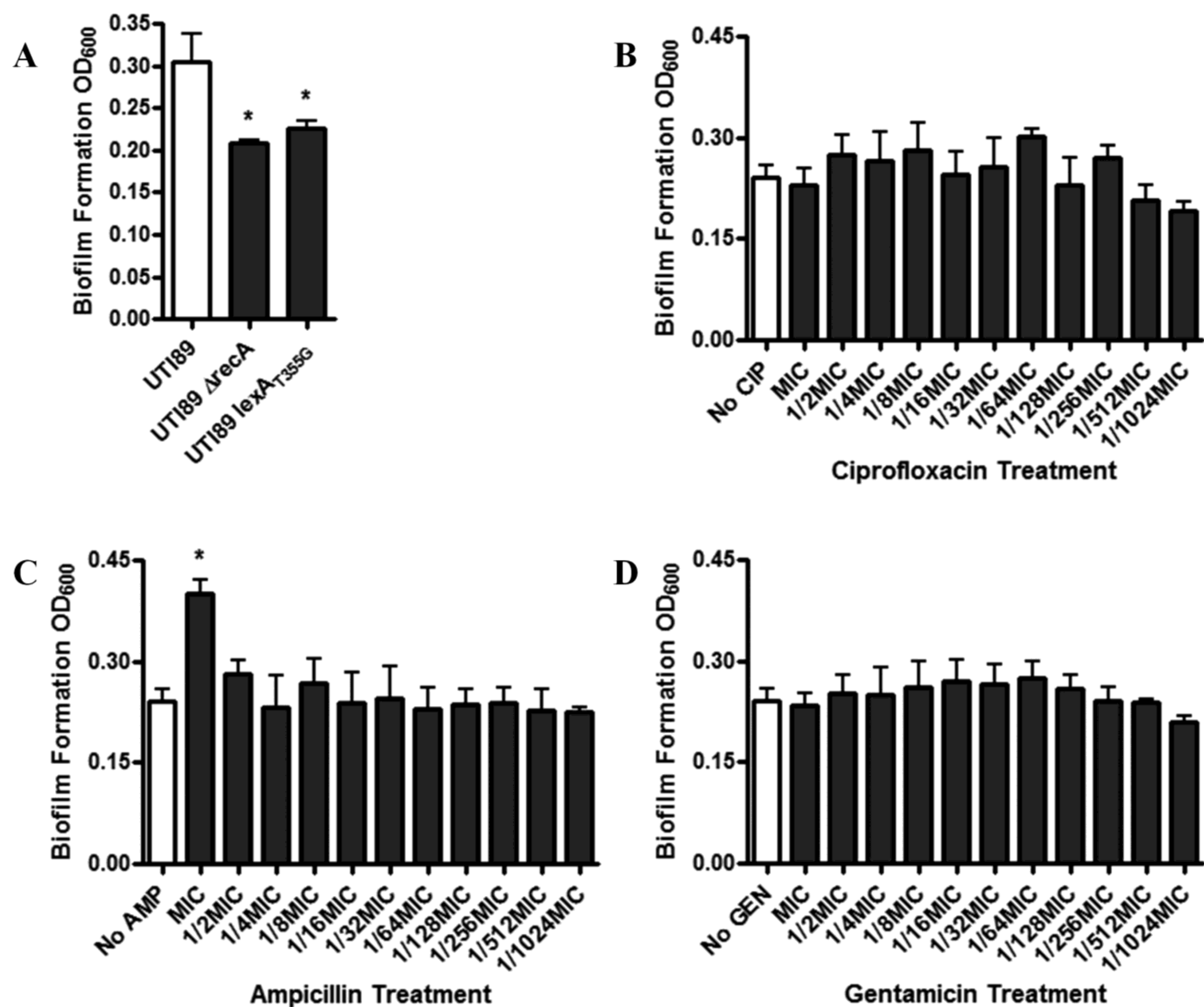


Figure 3.6 (continued)

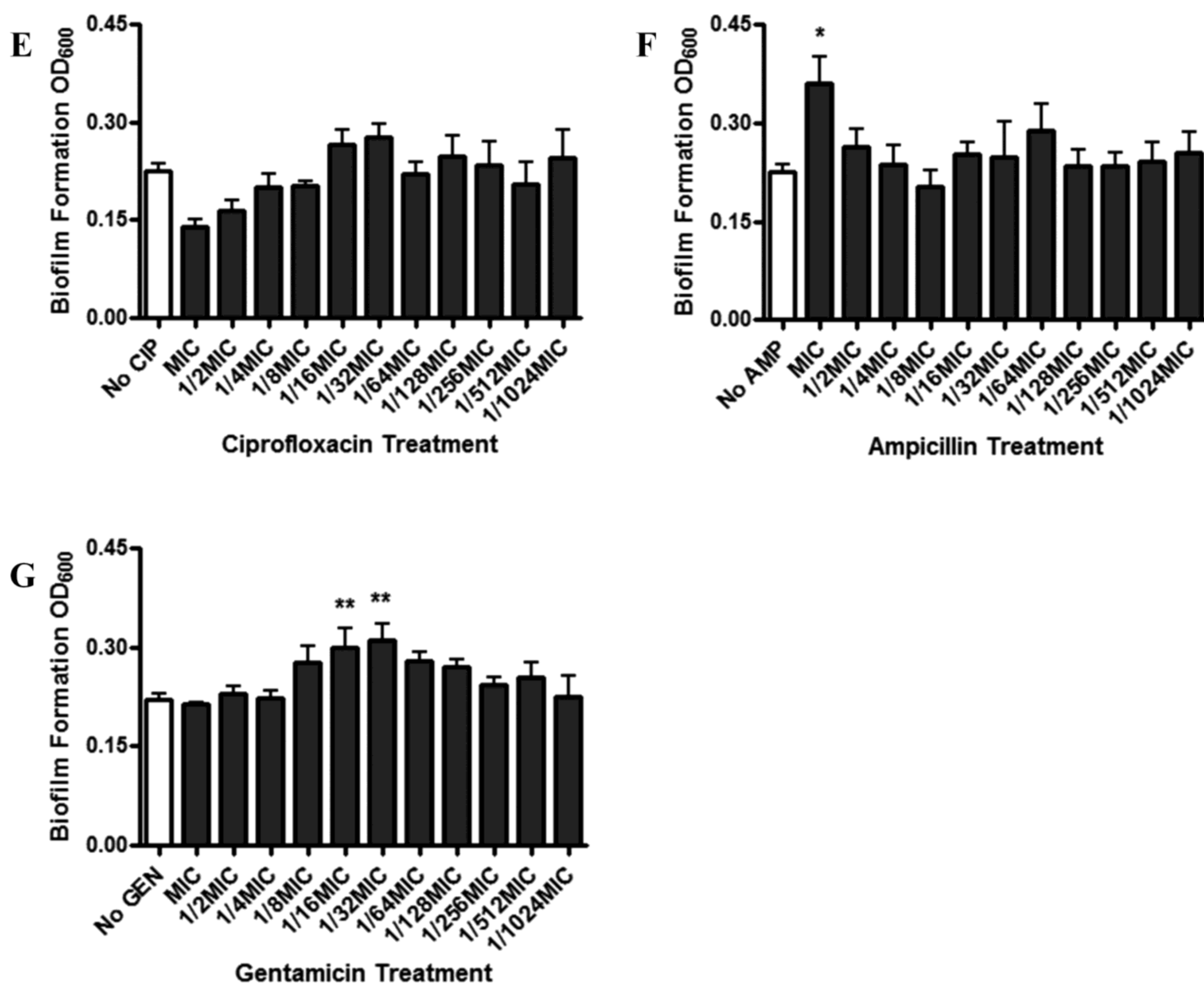
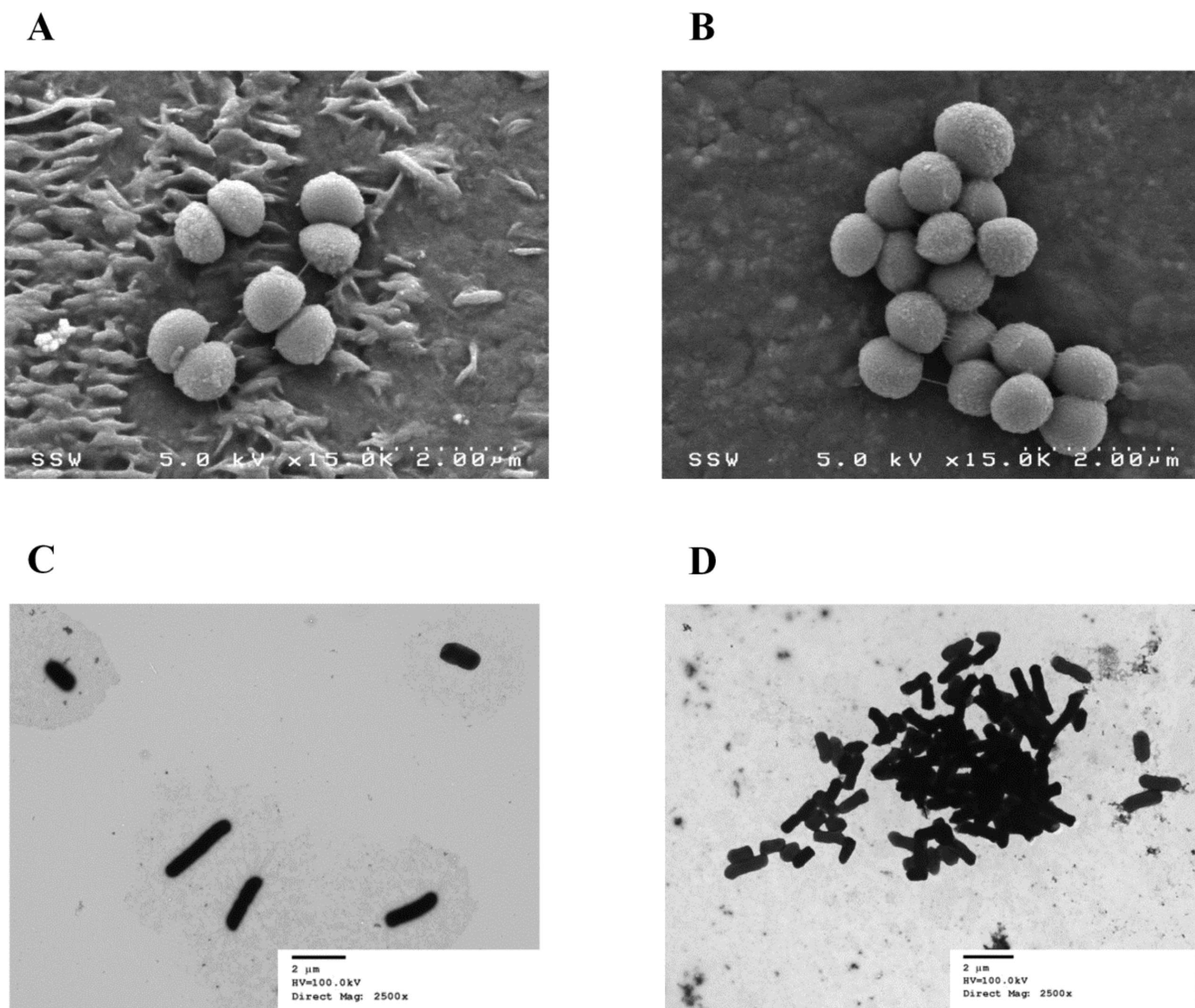


Figure 3.7. Representative images showing adherence and aggregation of *S. saprophyticus* 15305 (A, B; SEM), and *E. coli* UTI89 (C, D; TEM), and the effect of 1/4MIC ciprofloxacin treatment (B, D) on these processes.

Figure 3.7



S. saprophyticus 15305 aggregation was assessed using the glass microscope slide technique previously described. As expected from previous observations, antibiotic-priming significantly increased planktonic aggregate size (Figure 3.8A). Ciprofloxacin, ampicillin and gentamicin increased aggregate size from a control median of 6 to 9, 8, and 8 organisms/aggregate respectively ($P < 0.05$; $P < 0.01$). Inability to distinguish individual *E. coli* UTI89 organisms from one-another necessitated the use of TEM for accurate aggregation quantification. Similar to *S. saprophyticus* 15305, *E. coli* UTI89 was treated with 1/4MIC antibiotics for one hour prior to fixation and enumeration. Subsequent visualization demonstrated that like *S. saprophyticus* 15305, *E. coli* UTI89 aggregates in the presence of sub-inhibitory antibiotics (Figure 3.8B). Aggregate size increased from a control median of 3 to 6 organisms/aggregate for ciprofloxacin, ampicillin and gentamicin treatment groups ($P < 0.01$).

3.1.6 Evaluating the ability of antibiotics to induce *S. saprophyticus* urothelial cell adherence and internalization

Having demonstrated that antibiotic-primed uropathogens more readily adhere to abiotic surfaces, it was postulated that the same outcome would occur if urothelial cells were used as the adhering substrate. Experiments were conducted to assess the ability of antibiotics to improve *S. saprophyticus* 15305 adherence to both 5637 bladder and A498 kidney cell lines. These cell lines were selected for their similarity to normal urinary epithelium tissue. All antibiotics induced increased adherence to both cell lines, with ciprofloxacin, ampicillin and gentamicin improving adherence to 5637 bladder cells 12.8, 7.4, and 7.0-fold (Figure 3.9A), and 10.7, 11.4, and 9.7-fold in A498 kidney cells respectively ($P < 0.05$; $P < 0.01$) (Figure 3.9C). Antibiotic-dependent modulation of adherence also resulted in a larger intracellular reservoir for both cell types. Ciprofloxacin, ampicillin and gentamicin increased the intracellular population in 5637 bladder cells by 5.6, 6.6, and 8.3-fold respectively ($P < 0.01$) (Figure 3.9B). Similarly, ciprofloxacin and ampicillin both increased internalization 3.4-fold ($P < 0.05$), while gentamicin resulted in a 4.4-fold increase in A498 kidney cells ($P < 0.01$) (Figure 3.9D).

Figure 3.8. Average planktonic cluster size (bacteria/aggregate) of *S. saprophyticus* 15305 (A) and *E. coli* UTI89 (B) following exposure to either 1/4MIC ciprofloxacin, ampicillin, gentamicin, or no antibiotic. Means from at least three independent experiments are shown with significance. Significance was determined using one-way ANOVA and Dunnett's multiple comparison test (*, $P < 0.05$; **, $P < 0.01$).

Figure 3.8

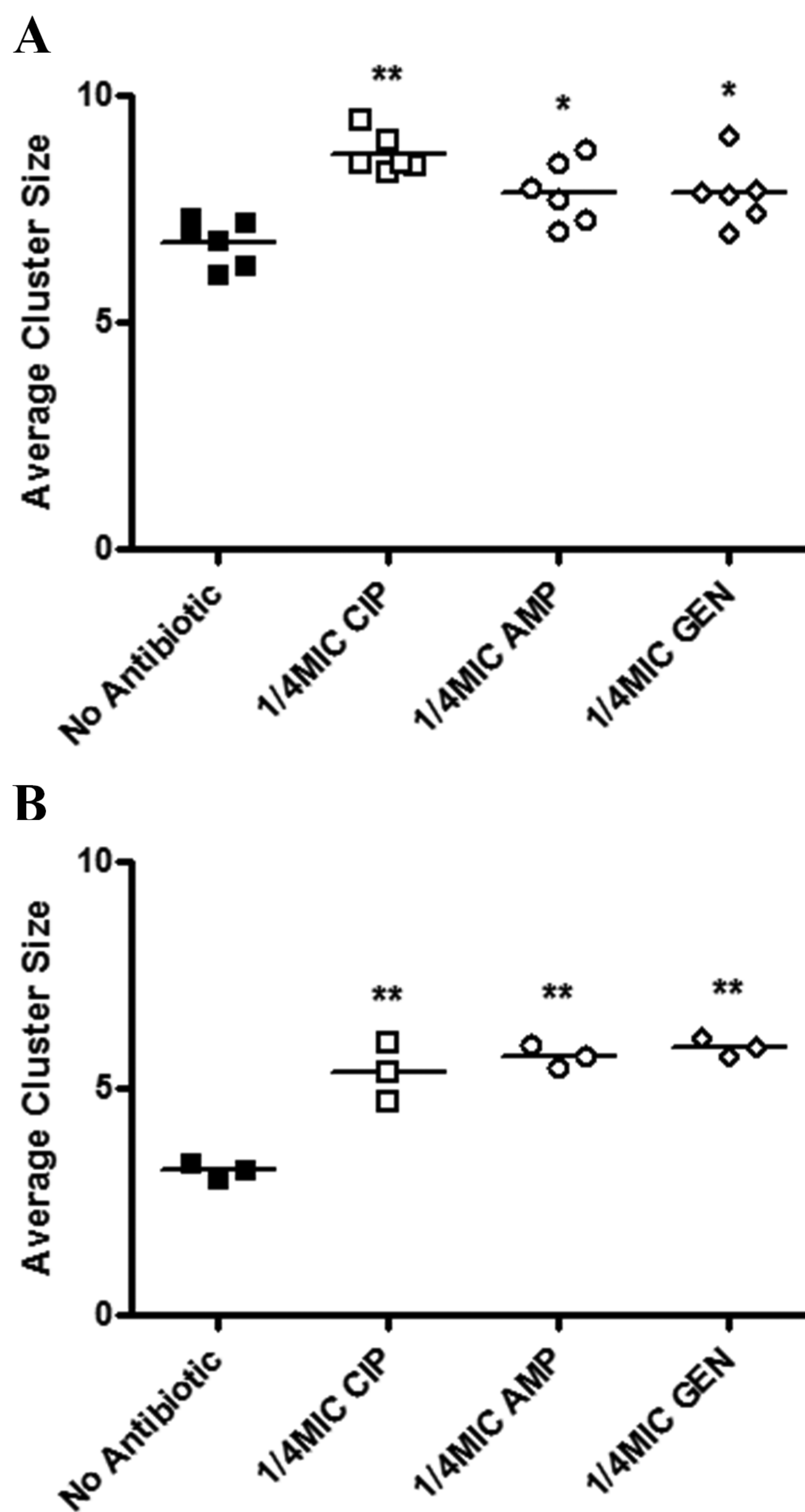
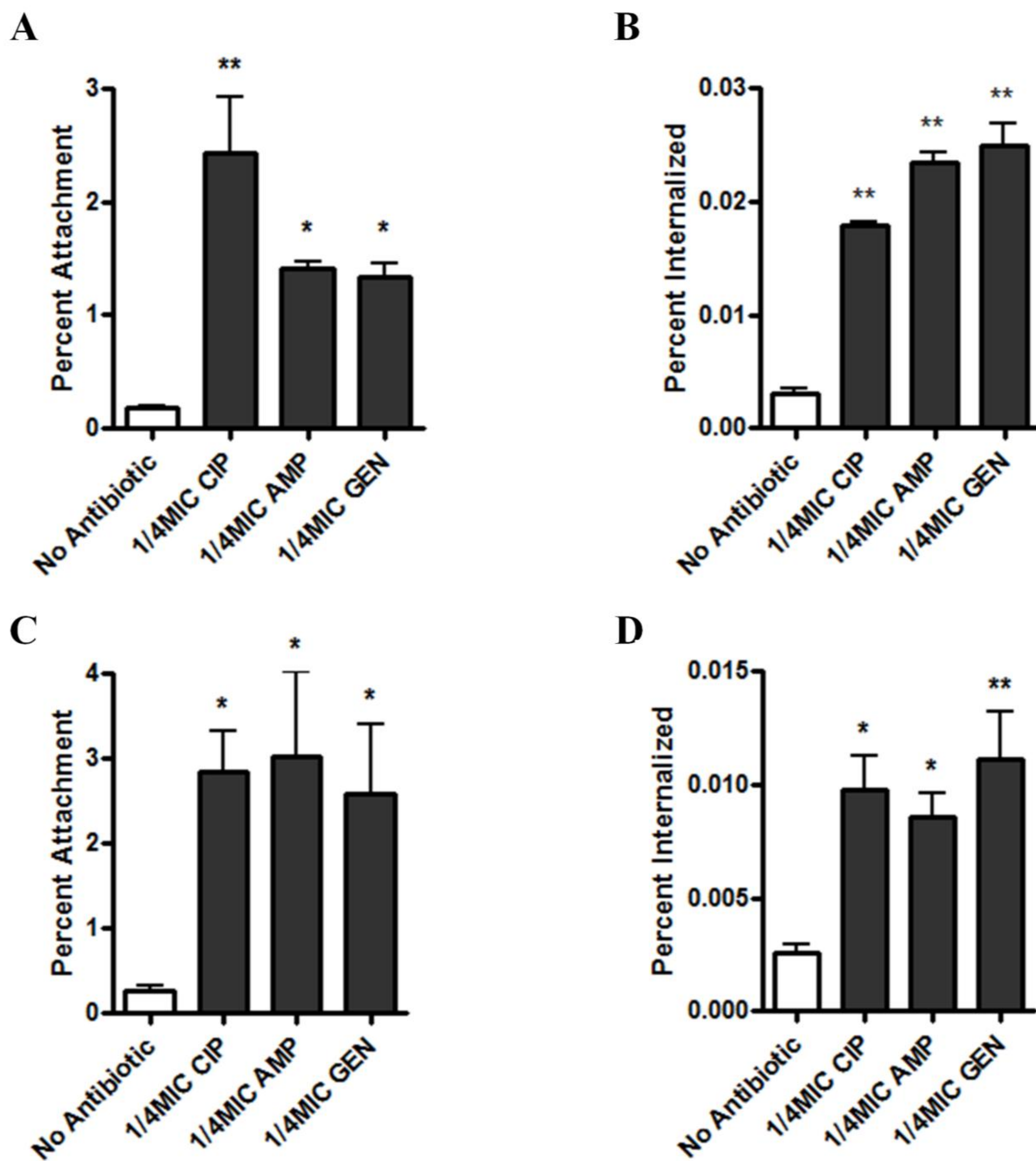


Figure 3.9. The effect of antibiotics on *S. saprophyticus* 15305 adherence (A, C) and internalization (B, D) to 5637 bladder (A, B) and A498 kidney cells (C, D). Means from at least three independent experiments are shown with significance. Significance was determined using one-way ANOVA and Dunnett's multiple comparison test (*, $P < 0.05$; **, $P < 0.01$).

Figure 3.9



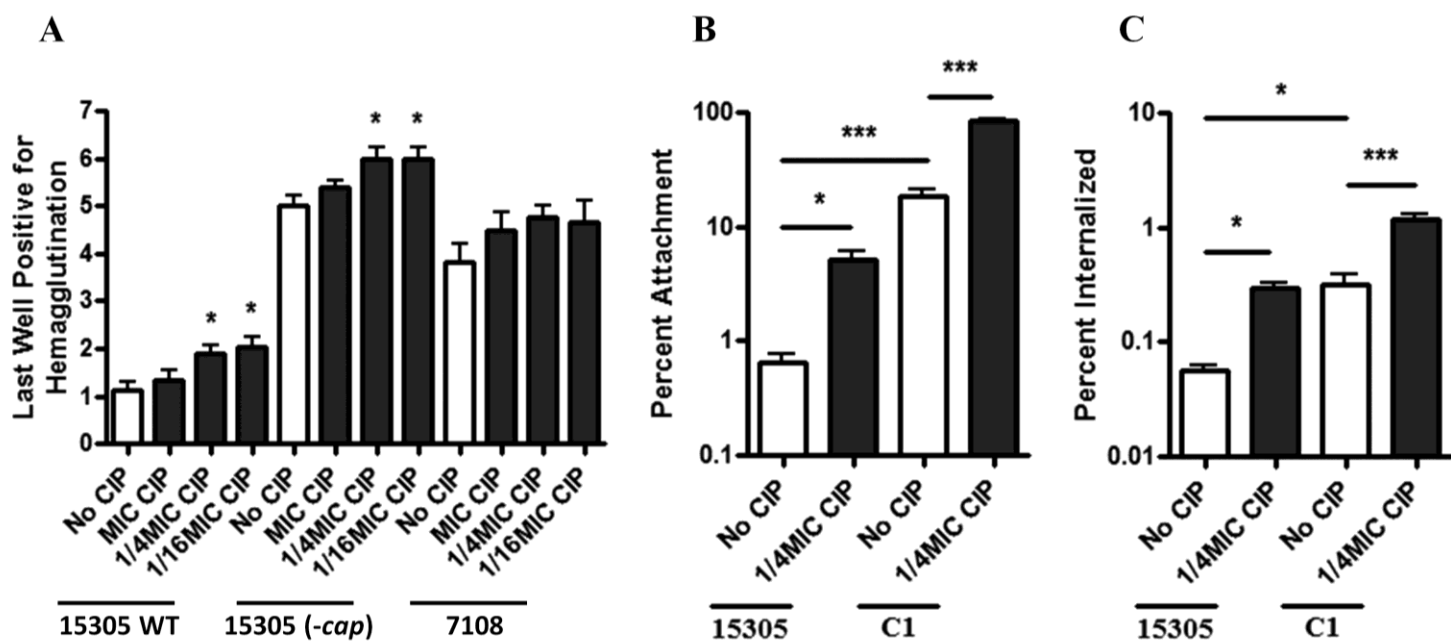
3.1.7 Assessing the contribution of adhesin upregulation to improved adhesion and internalization in antibiotic-primed *S. saprophyticus*

Antibiotic-dependent changes in adherence were much more substantial when biotic surfaces were used compared to abiotic urological device materials. This is likely not solely attributable to changes in the surface chemistry of *S. saprophyticus* 15305 exposed to antibiotic. Rather, the dramatic increase in relative adherence was postulated to be due to the improved expression of adhesins on the *S. saprophyticus* 15305 surface. As UafA is the most critical urothelial adhesion factor for this organism, its modulation was investigated. Hemagglutination experiments suggested that UafA is in part responsible for the increased adherence following ciprofloxacin therapy. The results demonstrate a dose-dependent effect, with 1/16MIC ciprofloxacin levels inducing a 45.0% increased level of hemagglutination ($P < 0.05$) (Figure 3.10A). The differences found between hemagglutination by wild-type *S. saprophyticus* 15305 and the acapsular C1 strain, demonstrate the blocking effect of the robust capsule. However, the greater hemagglutination following ciprofloxacin-priming in the absence of capsule, suggests that UafA surface expression was enhanced by antibiotics and not simply due to capsule downregulation. This effect was also observed in *S. saprophyticus* 7108, suggesting ciprofloxacin might have this affect across this species.

Adherence capacity to 5637 bladder cells following ciprofloxacin treatment was also compared between wild-type and acapsular strains of *S. saprophyticus*. As predicted, ciprofloxacin increased adhesion in both strains. These results further demonstrated that increased adhesion is likely due to UafA upregulation, not capsule suppression. Total wild-type adherence was increased from 0.7% to 5.2% for control and ciprofloxacin treatment groups respectively ($P < 0.05$), while adherence increased from 18.4% to 83.7% in the acapsular strain ($P < 0.001$) (Figure 3.10B). Internalization was also found to significantly increase in the absence of capsule, but in the presence of sub-inhibitory ciprofloxacin. Total wild-type internalization was improved from 0.057% to 0.294% for control and ciprofloxacin treatment groups respectively ($P < 0.05$), while internalization was increased from 0.323% to 1.188% in the acapsular strain ($P < 0.001$) (Figure 3.10C).

Figure 3.10. UafA-dependent hemagglutination of *S. saprophyticus* 15305, 7108, and the acapsular C1 strain following exposure to various concentrations of ciprofloxacin (A), and effect on 5637 bladder cell adherence (B) and internalization (C). Means from at least three independent experiments are shown with significance. Significance was determined using one-way ANOVA and Bonferroni's multiple comparison test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 3.10



3.1.8 Evaluating the capacity of sub-inhibitory antibiotics to induce adhesin expression in *E. coli*

The results obtained for *S. saprophyticus* entertained the idea that *E. coli* might also increase biofilm forming capacity following antibiotic-priming by upregulating critical surface adhesins. As type 1 fimbriae have been demonstrated to be critical in urothelial colonization, invasion, and UTI pathogenesis, antibiotic-dependent modulation of this adhesin was postulated to contribute to biofilm formation. Type 1 fimbriae-dependent hemagglutination was assessed over 24 hours and the results showed that expression increased over time and in response to 1/4MIC and 1/16MIC ciprofloxacin ($P < 0.05$; $P < 0.01$; $P < 0.001$) (Figure 3.11A).

Previous results suggest that the SOS response might partially contribute to *E. coli* UTI89 biofilm formation when antibiotics are applied. Thus, it was reasonable to postulate an SOS-dependent role in type 1 fimbriae regulation in *E. coli* UTI89. This was explored using SOS-deficient *E. coli* UTI89 strains PAS0209 and PAS0211. Baseline type 1 fimbriae expression levels were not significantly changed in the SOS-deficient strains (Figure 3.11B). Only wild-type *E. coli* UTI89 significantly increased hemagglutination following exposure to 1/4MIC ciprofloxacin, ampicillin, and gentamicin ($P < 0.05$), leaving SOS-deficient strains unaffected by antibiotic treatment.

3.1.9 Assessing the influence of sub-inhibitory antibiotics on *E. coli* phase variation as a mechanism of type 1 fimbriae expression

The mechanisms by which antibiotics improve adhesin expression in *E. coli* and *S. saprophyticus* are unclear. However regulation of type 1 fimbriae is phase variable, and the proportion of *E. coli* in phase ON or OFF orientation can be measured using PCR of the *fim*-switch, with restriction digestion of amplicons yielding 489 and 359 base-pair products for phase ON and OFF respectively (Figure 3.12A). Combined with densitometric analysis, these bands can accurately elucidate the relative gene expression of type 1 fimbriae in different conditions.

Figure 3.11. Type I fimbriae-dependent hemagglutination of *E. coli* UTI89 exposed to various subinhibitory concentrations of ciprofloxacin over time (A). Effect of 1/4MIC of various antibiotics on hemagglutination of wild-type and SOS-deficient *E. coli* strains (B). Means from at least three independent experiments are shown with significance. Significance was determined using one-way ANOVA and Bonferroni's multiple comparison test (ns = not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 3.11

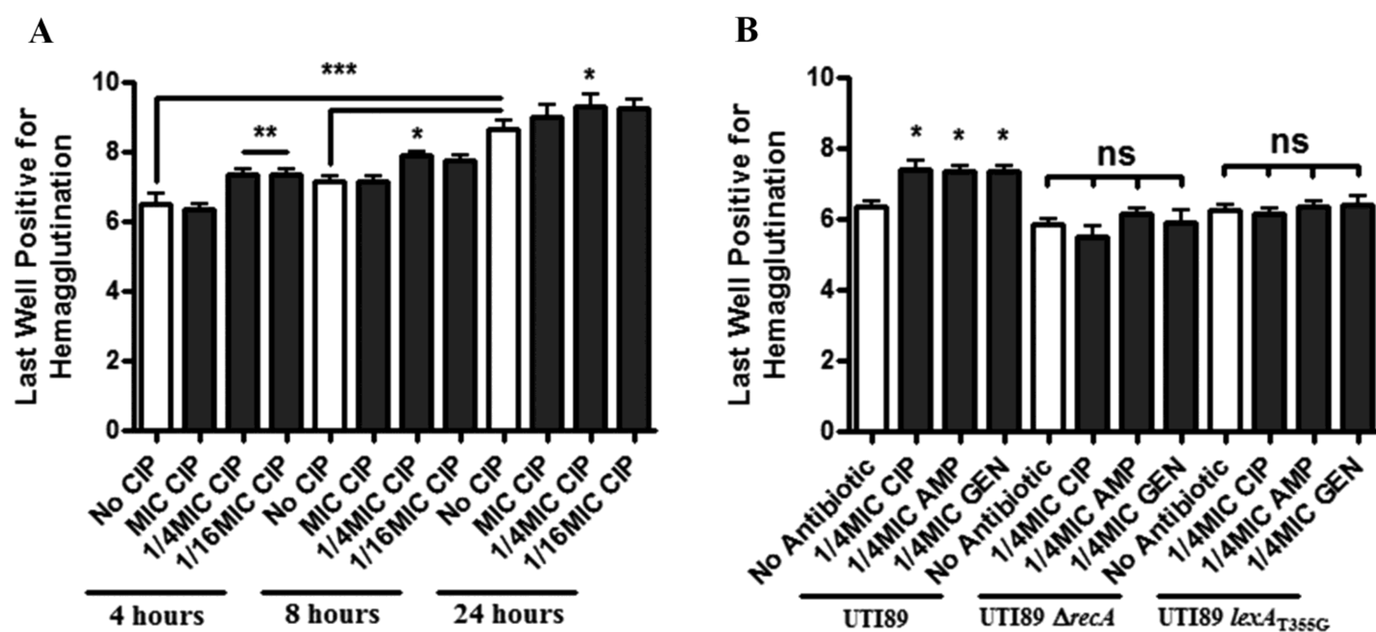
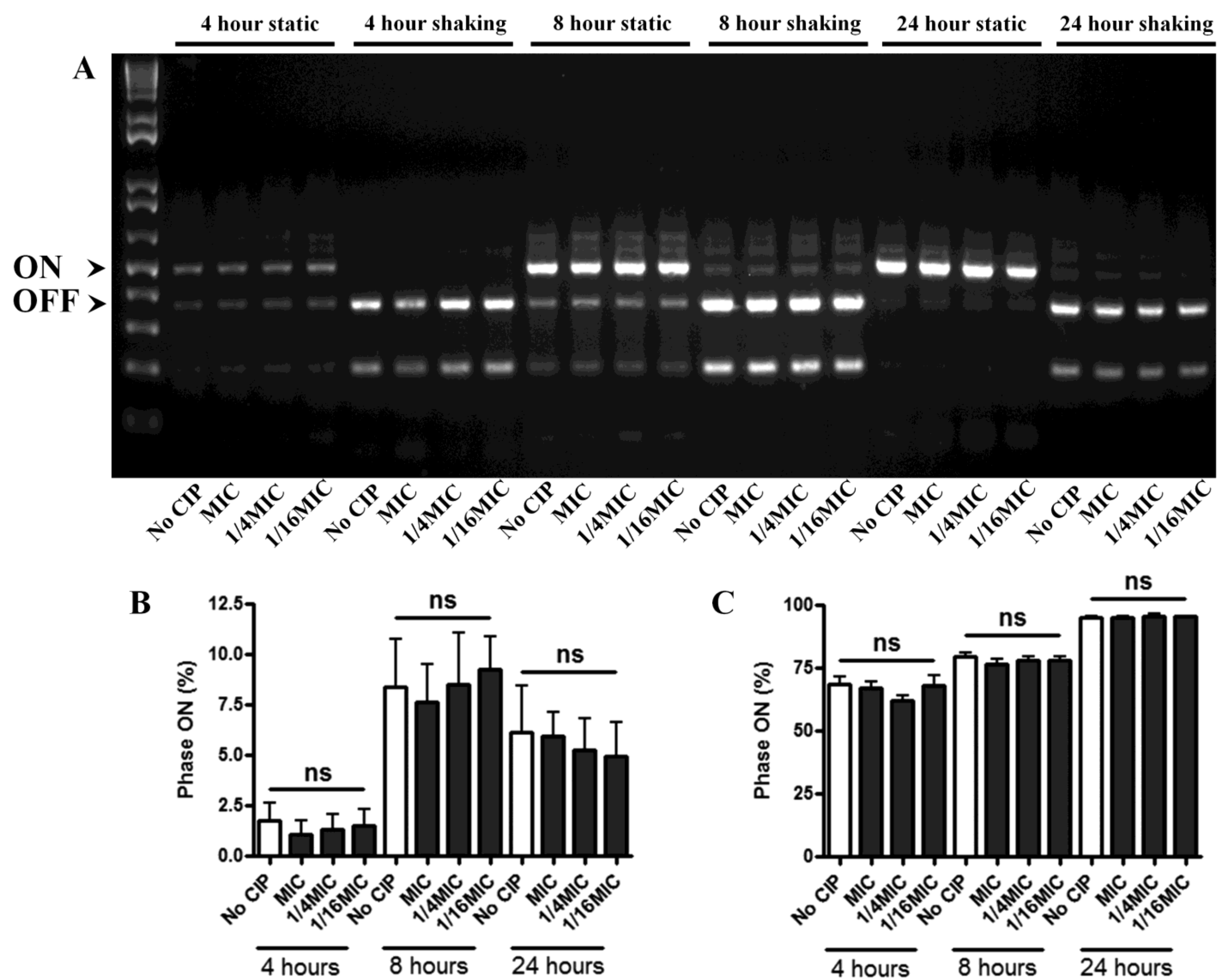


Figure 3.12. Time-course indicating Type I fimbriae gene ON/OFF phase changes in shaking or static cultures of *E. coli* UTI89 following exposure to various subinhibitory concentrations of ciprofloxacin (A). Densitometric gel analysis indicates the percentage of *E. coli* UTI89 with Type I fimbriae in the phase ON orientation during shaking (B) and static (C) growth. Means from at least four independent experiments are shown with significance. Significance was determined using one-way ANOVA and Bonferroni's multiple comparison test (ns = not significant).

Figure 3.12



Surprisingly, no significant changes were observed in OFF-ON phase variation in either shaking (Figure 3.12B) or static (Figure 3.12C) cultures at any time point with any sub-inhibitory ciprofloxacin treatment. At this time it is unclear how antibiotics regulate the expression of type 1 fimbriae in *E. coli* UTI89.

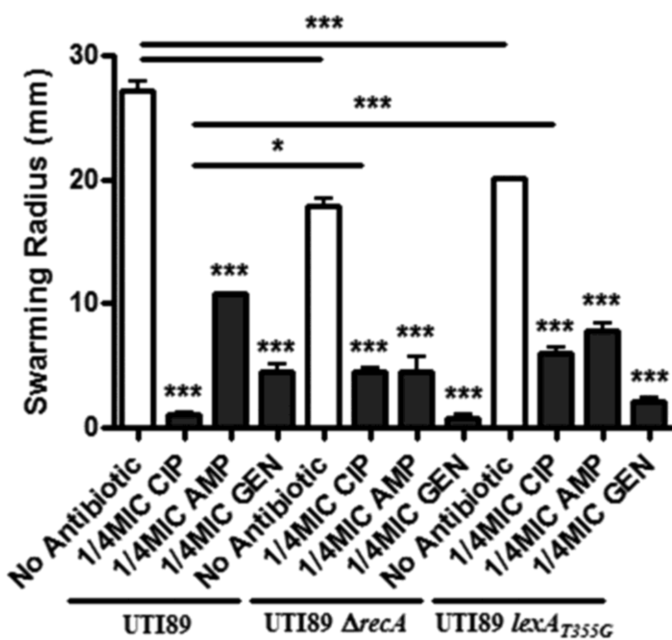
3.1.10 Evaluating the effect of sub-inhibitory antibiotics on flagellar activity in *E. coli*

Sub-inhibitory antibiotics appear to signal uropathogens to upregulate factors important to adherence and subsequently biofilm formation. Importantly, confounding factors which promote motility would ideally be suppressed in order to achieve a fully sedentary, adherent state. Thus, it is reasonable to postulate that flagellar activity would be suppressed following the application of sub-inhibitory antibiotics. *E. coli* UTI89 and SOS-deficient variants PAS0209 and PAS0211 were spot plated on soft-agar plates in the presence or absence of sub-inhibitory antibiotics (Figure 3.13A). These strains were also pre-treated with sub-inhibitory antibiotics prior to loading on untreated soft-agar plates to assess antibiotic changes to flagella regulation during planktonic phase growth (Figure 3.13B). Interestingly, SOS-deficient strains demonstrated less motility compared to wild-type *E. coli* UTI89 in both conditions. In general, sub-inhibitory antibiotics suppressed motility in all strains tested, with the exception of ciprofloxacin which significantly upregulated swarming in the $lexA_{T355G}$ *E. coli* PAS0211 strain (Figure 3.13A). This finding was recapitulated in antibiotic-primed organisms, with wild-type *E. coli* UTI89 demonstrating a greater swarming deficiency following ciprofloxacin treatment than its SOS-deficient counterparts (Figure 3.13B).

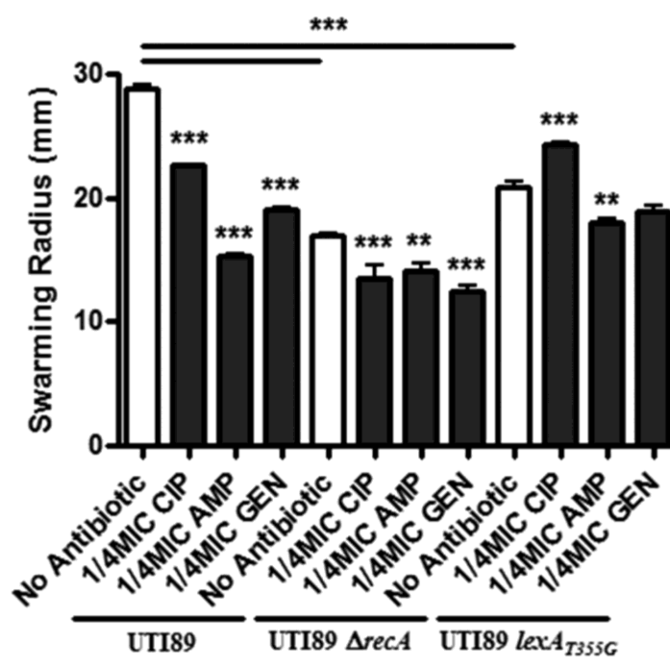
Figure 3.13. Swarming motility of wild-type and SOS-deficient strains of *E. coli* UTI89 in the presence of sub-inhibitory antibiotics. Strains were either plated directly onto soft-agar containing antibiotic (A), or pre-treated with antibiotics for 4 hours prior to plating on unloaded soft-agar (B). Means from at least three independent experiments are shown with significance. Significance was determined using one-way ANOVA and Bonferroni's multiple comparison test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 3.13

A



B



3.1.11 Assessing sub-inhibitory antibiotic-depended changes in gene expression in both *E. coli* UTI89 and *S. saprophyticus* 15305

RNA sequencing was performed in order to better understand the pathways involved in antibiotic tolerance and signalling, and to corroborate data generated using phenotypic assays. Reads mapped to genomic coding regions were grouped by their annotated function in order to evaluate the differences between control (no antibiotic), and short (30 minute) or long (4-6 hour) 1/4MIC antibiotic treatment groups for both *E. coli* UTI89 and *S. saprophyticus* 15305. SEED Subsystem annotations were used to estimate expression differences for subsystem 4 functions using ALDEx and expressed in the form of strip plots where subsys4 functions were plotted by absolute fold-change between conditions. Analysis of differential expression of *E. coli* UTI89 genes (Figure 3.14A, B) at the broadest functional level (SEED subsystem 1) revealed high variation in responses between 30 minutes (Figure 3.14A) and 4 hours (Figure 3.14B) of sub-inhibitory ciprofloxacin exposure. Early responses in *E. coli* UTI89 are dominated by increased relative abundance of transcripts related to DNA metabolism. Long-term exposure revealed similar changes in DNA metabolism-related genes, in addition to increases in the relative abundance of transcripts related to virulence and phage-components. Transcripts related to stress, respiration, carbohydrate and amino acid metabolism were generally found at a lower relative abundance following long-term ciprofloxacin exposure.

The responses of *S. saprophyticus* 15305 to sub-inhibitory ciprofloxacin were markedly different compared to those of *E. coli* UTI89 (Figure 3.14C, D). Early (30 minutes) responses were dominated by increases in the relative abundance of transcripts related to protein, DNA, and carbohydrate metabolism (Figure 3.14C). After prolonged ciprofloxacin exposure (6 hours), stress response transcripts are abundant, in addition to those related to respiration, and protein and carbohydrate metabolism (Figure 3.14D). Despite ciprofloxacin's ability to induce DNA-related stress, there were few changes in elements related to DNA maintenance in *S. saprophyticus* 15305 compared to *E. coli* UTI89. In addition, overall changes in transcripts related to cellular activity (respiration

Figure 3.14. Subsystem 1 categories indicating enriched subsystem 4 functions for *E. coli* UTI89 (A, B) and *S. saprophyticus* 15305 (C, D) exposed to sub-inhibitory ciprofloxacin for either 30 minutes (A, C), 4 hours (B), or 6 hours (D). Each point represents a specific subsystem 4 function, and the magnitude in change is plotted on the x-axis relative to ciprofloxacin treatment conditions. Points are coloured cyan (relative transcript abundance decreased following treatment) or red (relative transcript abundance increased following treatment) if significantly different between conditions, while grey points are non-significant changes. A $\log_2$ median effect size of at least 1.5 was required for genes to be considered differentially expressed. In addition, a less than 1% overlap in the distributions between the two conditions was permitted for inclusion.

Figure 3.14

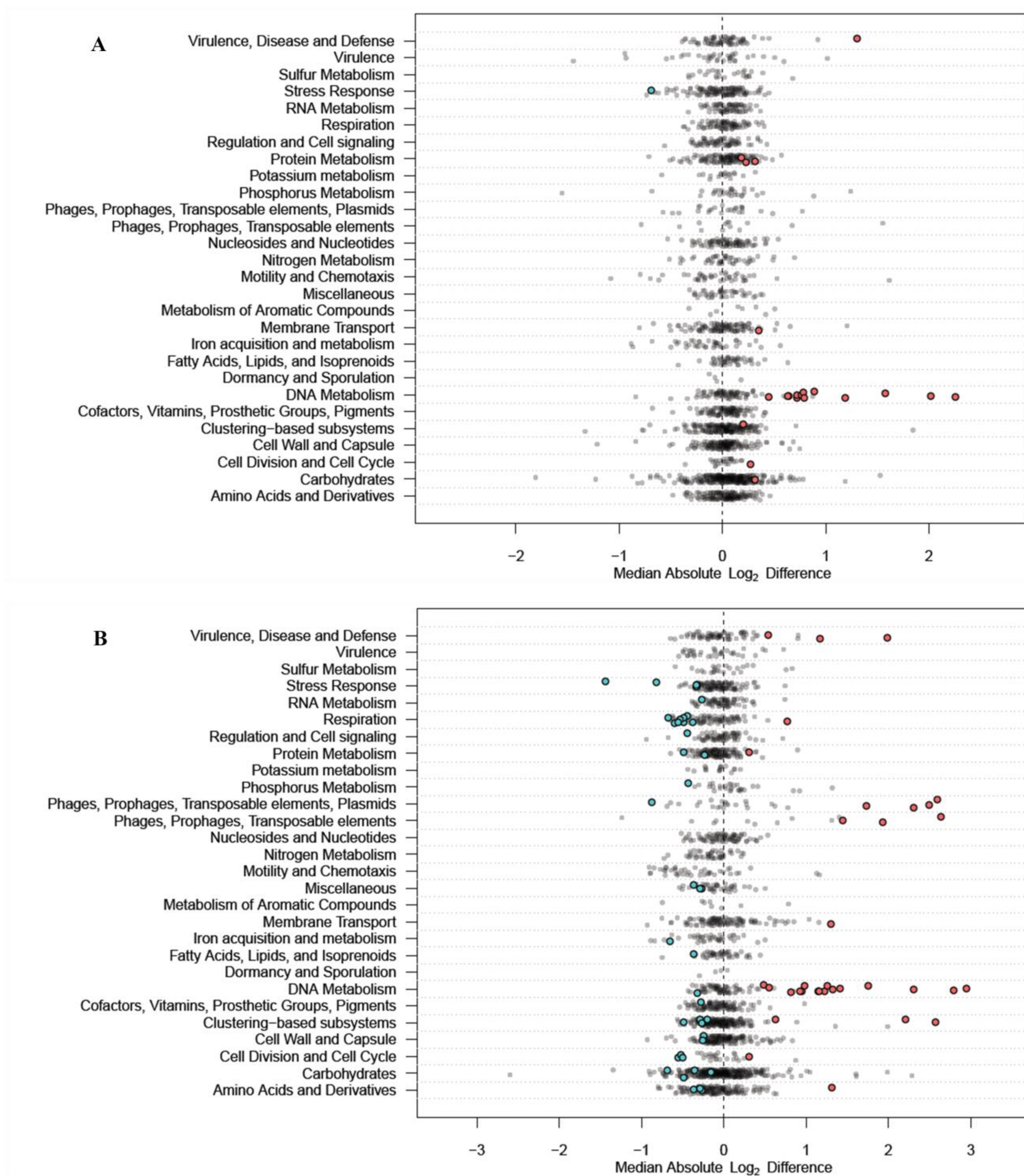
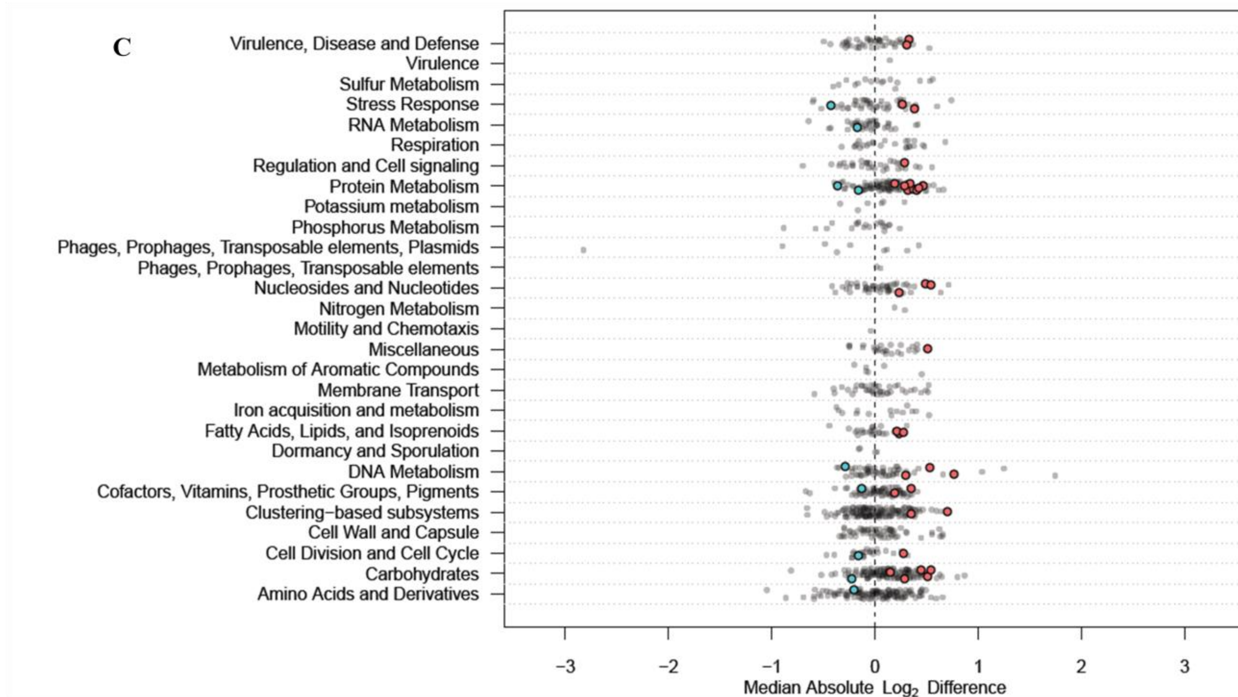
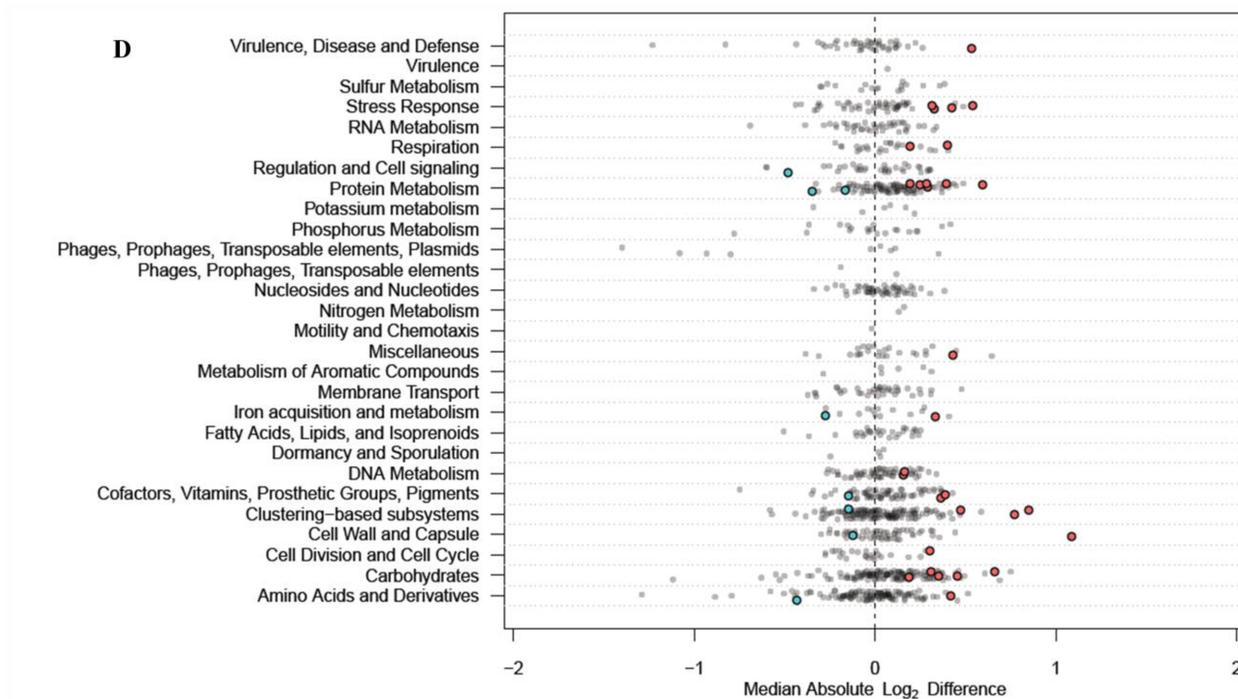


Figure 3.14 (continued)





and metabolism) were increased in *S. saprophyticus* 15305 following ciprofloxacin treatment while they were decreased in *E. coli* UTI89.

Assessing the changes in relative abundance of specific transcripts indicated several genes that might contribute to adherence, invasion, and biofilm formation in both uropathogens. The majority of early *E. coli* UTI89 responses were dominated by stress-inducible elements including damage-inducible (*din*) genes (*dinG*, *dinF*, *dinP*, *dinD*), nucleotide excision repair (NER) factors (*uvrB*, *uvrD*), and SOS regulon-associated components *sulA*, *lexA*, and *recN* (Table 3.2). Late responses in this uropathogen were comparatively more diverse, and included increased relative abundance in the aforementioned transcripts, in addition to several phage components (*kil2*, *erf*, *ninE2*, *ninB2*, *ninB1*, *ral*, *cro*, etc) (Table 3.3). Conversely, transcripts related to cell division (*minE*, *minD*, *minC*), and metabolism regulatory elements (*yjdE*, *lacY*, *nuoM*, *sdhA*, *nuoF*, *nuoJ*) were relatively decreased. Transcripts of the fimbrial repressor element *focX*, and the *luxR* homolog *sdiA* were significantly decreased 1.48 and 4.37-fold following 4 hours of sub-inhibitory ciprofloxacin treatment. Conversely, putative fimbrial FimH (*fmlD*) and *hek* [also known as heat resistant agglutinin-1 (Hra1)] adhesin transcripts were significantly more abundant 4.84 and 2.47-fold in the ciprofloxacin treatment group compared to control.

Early responses of *S. saprophyticus* 15305 to sub-inhibitory ciprofloxacin are characterized by decreased relative abundance of transcripts related to cell wall remodelling (SSP0843, SSP0988, SSP1294), and increases in transcripts related to protein stabilization and folding (*groEL*, SSP0296), cell division inhibition (SSP1593), metabolism (SSP0750, SSP2178, SSP1416, SSP0333, SSP1618, etc), exopolysaccharide synthesis (*capH*, *capL*), and general stress responses (SSP1024) (Table 3.4). In addition, an increased relative abundance of *recA* transcripts is indicative of DNA damage; however, late responses demonstrate that *S. saprophyticus* 15305 does not commit to a full-fledged SOS response during sub-inhibitory ciprofloxacin treatment as SOS regulon transcripts (*lexA*, NER components, error-prone polymerases, etc) are absent (Table 3.5). Rather, elements related to energy production and utilization (SSP1520, SSP1819, SSP2178, SSP1814, SSP1358, SSP0776, SSP1068, etc), protein stabilization (SSP0296, SSP1800, SSP1178, SSP1176, *dnaK*), and exopolysaccharide synthesis (SSP0769, *capH*)

are present at greater relative abundance. In addition to exopolysaccharide, increased presence of other virulence-related transcripts of unknown function included putative virulence factor B and hemolysin III homolog. Together, the divergent responses detailed by these transcriptomic data indicate that *E. coli* UTI89 and *S. saprophyticus* 15305 utilize distinct mechanisms to tolerant sub-inhibitory ciprofloxacin-dependent stress.

Table 3.2 Significant changes in differentially expressed transcripts - *E. coli* UTI89 control versus 1/4MIC ciprofloxacin (short-term exposure)

Gene	Fold Change (log_2)	Median Effect Size	Functional Annotation
UTI89_C1489	-0.724	-2.506	putative phage protein
cspE	-0.692	-2.523	cold shock protein
tqsA	-0.657	-2.375	pheromone autoinducer 2 transporter
UTI89_C4928	-0.593	-1.923	unknown
UTI89_C5141	-0.473	-1.83	unknown
ydfZ	-0.395	-1.765	putative selenium-binding protein
lysU	0.183	2.229	lysyl-tRNA synthetase
ydeN	0.2	2.107	sulfatase
selD	0.23	1.858	selenophosphate synthetase
ftsK	0.272	1.626	DNA translocase FtsK
manX	0.273	1.539	PTS system mannose-specific transporter
UTI89_C0964	0.286	1.873	putative phage regulator Cox
UTI89_C2924	0.287	1.555	unknown
pgk	0.318	1.741	phosphoglycerate kinase
selA	0.319	2.761	selenocysteine synthase
tatB	0.349	2.477	sec-independent translocase
int	0.421	2.904	integrase
uvrB	**0.45**	**1.719**	**excinuclease ABC subunit B**
uvrD	**0.629**	**2.117**	**DNA-dependent helicase II**
ruvB	**0.645**	**2.529**	**Holliday junction DNA helicase**
dinG	**0.72**	**3.94**	**ATP-dependent DNA helicase**
polB	**0.724**	**5.097**	**DNA polymerase II**
umuC	**0.763**	**3.12**	**DNA polymerase V subunit**
UTI89_C5142	0.764	4.032	unknown
ydjQ	**0.783**	**2.352**	**nucleotide excision repair endonuclease**
ruvA	**0.791**	**3.18**	**Holliday junction DNA helicase**
dinP	**0.892**	**4.023**	**DNA polymerase IV**
sulA	**0.998**	**2.459**	**SOS cell division inhibitor**
yebG	**1.156**	**2.754**	**DNA damage-inducible protein**
dinD	**1.159**	**2.447**	**DNA-damage-inducible protein**
recA	**1.184**	**2.78**	**DNA repair, SOS induction**
UTI89_C4473	1.221	3.011	putative lipase
dinF	**1.3**	**7.118**	**DNA-damage-inducible SOS response protein**
lexA	**1.334**	**2.877**	**repressor of LexA**
UTI89_C1922	1.371	2.442	contains a metal-dependent hydrolase
umuD	**1.58**	**3.75**	**DNA polymerase V subunit**
UTI89_C5143	**2.021**	**5.049**	**putative DinI**
recN	**2.255**	**7.558**	**recombination and repair protein**

Genes indicated in bold are putative SOS regulon-associated elements.

Table 3.3 Significant changes in differentially expressed transcripts - *E. coli* UTI89 control versus 1/4MIC ciprofloxacin (long-term exposure)

Gene	Fold Change (log_2)	Median Effect Size	Functional Annotation
adiA	-1.442	-1.986	biodegradative arginine decarboxylase
UTI89_C1489	-1.363	-3.627	putative phage protein
UTI89_C5141	-0.966	-3.129	unknown
UTI89_C0936	-0.953	-2.399	small terminase subunit of phage
UTI89_C0914	-0.88	-2.183	phage tail sheath protein
UTI89_C0938	-0.852	-3.202	phage capsid scaffolding protein
yjdE	-0.816	-1.872	arginine:agmatin antiporter
UTI89_C0915	-0.69	-2.872	phage tube protein FII
lacY	-0.683	-2.556	lactose permease
UTI89_C1011	-0.675	-2.931	putative iron-sulfur protein, 2Fe-2S clusters
UTI89_C5140	-0.675	-2.452	unknown
nuoM	-0.674	-1.896	NADH dehydrogenase subunit M
ycdB	-0.657	-2.834	unknown
sdhA	-0.652	-1.764	succinate dehydrogenase flavoprotein subunit
ycgE	-0.635	-2.305	HTH-transcriptional repressor
UTI89_C3113	-0.625	-2.276	putative lipoprotein NlpD
UTI89_C2270	-0.598	-2.1	putative phospholipase
UTI89_C1363	-0.595	-2.616	putative prophage protein
focX	-0.562	-2.156	repressor of S fimbrial adhesin
minE	-0.556	-3.683	cell division topological specificity factor
nuoF	-0.551	-1.595	NADH dehydrogenase I subunit F
nuoJ	-0.538	-2.15	NADH dehydrogenase subunit J
minD	-0.517	-2.349	cell division inhibitor
UTI89_C2959	-0.507	-3.758	putative prophage protein
minC	-0.499	-2.276	septum formation inhibitor
nuoN	-0.49	-6.227	NADH dehydrogenase subunit N
UTI89_C2766	-0.49	-1.517	putative acetyltransferase
msrB	-0.486	-2.088	methionine sulfoxide reductase B
dadA	-0.485	-2.048	D-amino acid dehydrogenase small subunit
ldcA	-0.484	-2.924	L,D-carboxypeptidase A
sdhB	-0.478	-1.822	succinate dehydrogenase iron-sulfur subunit
UTI89_C0728	-0.476	-1.858	putative cytochrome d ubiquinol oxidase
UTI89_C0965	-0.453	-2.388	bacteriophage WPhi phage protein C
sdiA	-0.45	-2.255	putative quorum sensing homolog of LuxR
cyoB	-0.45	-1.926	cytochrome o ubiquinol oxidase subunit I
cydB	-0.446	-1.943	cytochrome d terminal oxidase polypeptide
ynjC	-0.442	-2.437	ABC transporter solute-binding protein
pntA	-0.43	-1.864	NAD(P) transhydrogenase subunit alpha
ycdW	-0.422	-2.437	glyoxylate/hydroxypyruvate reductase A
ompA	-0.395	-2.505	outer membrane protein A
UTI89_C1090	-0.389	-3.108	unknown
UTI89_C1960	-0.386	-2.637	putative NAD(P)H nitroreductase
UTI89_P009	-0.378	-3.015	ubiquinone
znuA	-0.375	-2.107	high-affinity periplasmic zinc transporter
UTI89_C0750	-0.374	-2.939	homeobox protein
UTI89_C1017	-0.374	-1.866	unknown
accB	-0.373	-3.511	acetyl-CoA carboxylase biotin carboxyl carrier

Table 3.3 (continued)

Gene	Fold Change (log_2)	Median Effect Size	Functional Annotation
ybbM	-0.372	-1.949	metal resistance protein
gdhA	-0.369	-1.895	glutamate dehydrogenase
dhaK	-0.363	-2.752	dihydroxyacetone kinase subunit
ydiA	-0.361	-1.797	unknown
fumA	-0.359	-2.513	class I fumarase
cspD	-0.333	-1.691	stationary phase/starvation regulator
uspC	-0.329	-2.774	universal stress protein
cstA	-0.326	-2.034	carbon starvation protein A
mukE	-0.323	-1.946	condensin subunit E
narX	-0.301	-2.111	nitrate/nitrite sensor protein
yecA	-0.3	-1.9	unknown
gltP	-0.29	-2.84	glutamate/aspartate:proton symporter
UTI89_C0891	-0.288	-2.945	CRISPR helicase Cas3/CRISPR protein Cas1
metH	-0.286	-1.919	B12-dependent methionine synthase
UTI89_C0833	-0.28	-2.111	hemin-binding lipoprotein
folX	-0.276	-2.138	pterin biosynthesis
ybbL	-0.272	-1.997	ABC transporter ATP-binding protein
yjeQ	-0.272	-1.994	ribosome-associated GTPase
mfd	-0.268	-1.821	transcription-repair coupling factor
artP	-0.268	-1.656	arginine transporter ATP-binding subunit
ompC	-0.256	-1.746	porin
UTI89_C1133	-0.251	-3.115	putative HcpC beta-lactamase precursor
hlpA	-0.247	-1.799	periplasmic chaperone
pflB	-0.244	-1.563	putative formate acetyltransferase 1
UTI89_C2496	-0.242	-1.872	putative outer membrane protein
ydhO	-0.234	-1.742	putative lipoprotein
asnC	-0.234	-1.707	asparaginyl-tRNA synthetase
UTI89_C1472	-0.226	-1.859	unknown
cld	-0.224	-1.839	length regulator of LPS O-antigen chains
yfhM	-0.202	-1.85	putative lipoprotein
UTI89_C3005	-0.189	-2.077	unknown
sfcA	-0.158	-2.021	malate dehydrogenase
ftsK	0.304	1.962	DNA translocase FtsK
selA	0.306	2.218	selenocysteine synthase
osmB	0.383	2.223	lipoprotein
UTI89_C1937	0.395	1.826	unknown
spy	0.432	1.99	spheroplast protein Y
uvrB	**0.483**	**3.317**	**excinuclease ABC subunit B**
int	0.518	2.698	integrase
ibpA	0.534	1.501	heat shock protein
endA	0.553	1.934	endonuclease I
yebF	0.623	1.933	unknown
UTI89_C2389	0.624	1.999	putative molybdate metabolism regulator
yfdQ2	0.703	1.94	unknown
ydeP	0.772	2.381	oxidoreductase
uvrD	**0.812**	**2.591**	**DNA-dependent helicase II**
UTI89_C2643	0.814	2.455	gene 16-like, phage DNA transfer protein

Table 3.3 (continued)

Gene	Fold Change (log_2)	Median Effect Size	Functional Annotation
uvrA	**0.919**	**1.976**	**excinuclease ABC subunit A**
ruvB	**0.949**	**4.379**	**Holliday junction DNA helicase**
UTI89_C4650	0.964	1.74	putative SoxR
ruvA	**0.985**	**7.498**	**Holliday junction DNA helicase**
polB	**1.149**	**4.389**	**DNA polymerase II**
rmuC	**1.151**	**1.688**	**DNA recombination protein**
UTI89_C1713	1.151	3.164	HTH/AraC transcriptional regulator
UTI89_C1753	1.158	2.055	maltoporin-like protein
ibpB	1.171	2.118	heat shock chaperone
yfdQ1	1.177	2.653	unknown
dinG	**1.221**	**6.389**	**ATP-dependent DNA helicase**
UTI89_C1023	1.229	2.915	unknown
UTI89_C5093	1.256	2.315	unknown
ydjQ	**1.257**	**2.289**	**nucleotide excision repair endonuclease**
UTI89_C0582	1.276	1.653	unknown
UTI89_C2644	1.302	3.853	gene 20-like, phage DNA transfer protein
hek	1.304	3.475	Hek adhesin/virulence factor
asnA	1.306	2.394	asparagine synthetase AsnA
umuC	**1.318**	**6.519**	**DNA polymerase V subunit**
UTI89_P101	1.335	5.005	unknown
dinD	**1.365**	**4.762**	**DNA-damage-inducible protein**
UTI89_C2690	1.369	1.966	putative phage protein
dinP	**1.407**	**3.789**	**DNA polymerase IV**
UTI89_C2653	1.447	2.66	gene 1-like, phage portal protein
UTI89_P099	1.468	2.501	unknown
sulA	**1.499**	**4.158**	**SOS cell division inhibitor**
UTI89_C5142	1.585	6.784	unknown
UTI89_C2647	1.593	2.296	gene 26-like, phage head assembly protein
hkaH	1.598	1.913	unknown
UTI89_C4473	1.611	3.933	putative lipase
UTI89_P091	1.64	2.56	unknown
UTI89_C2675	1.653	1.992	CII, involved in the initiation of lysogeny
UTI89_C2648	1.681	3.111	gene 10-like bacteriophage DNA stabilizer
UTI89_C5096	1.735	2.274	regulatory phage protein
UTI89_C2649	1.744	2.331	putative P22 tail-4 accessory factor
recA	**1.751**	**3.75**	**DNA repair, SOS induction**
UTI89_C2646	1.787	2.712	gene 14-like, putative phage protein
UTI89_C5091	1.792	1.892	putative phage protein
UTI89_C0801	1.808	2.066	unknown
UTI89_C2654	1.865	2.878	gene 2-like, putative phage terminase
UTI89_C2657	1.871	2.54	putative phage regulatory protein Rha
cro	1.93	2.69	phage Cro protein
UTI89_C2651	1.932	2.896	putative phage capsid/coat protein
UTI89_C2660	1.95	3.036	gene R-like, bacteriophage lysozyme
UTI89_C2672	1.97	2.46	bacteriophage Nil2 gene P DnaB analogue
dinF	**1.988**	**5.541**	**DNA-damage-inducible SOS response protein**
yahM	1.996	2.198	unknown

Table 3.3 (continued)

Gene	Fold Change (log_2)	Median Effect Size	Functional Annotation
UTI89_C1922	2	6.13	contains a metal-dependent hydrolase
lexA	**2.03**	**5.635**	**repressor of LexA**
yebG	**2.081**	**5.7**	**DNA damage-inducible protein**
UTI89_C2655	2.091	2.899	putative phage terminase
hkbD	2.094	2.575	unknown
UTI89_C2678	2.121	2.269	regulatory phage protein
ral	2.145	2.189	bacteriophage lambda restriction inhibitor
UTI89_C2692	2.147	1.688	putative prophage protein
UTI89_C2691	2.151	1.632	putative phage protein
UTI89_C2656	2.157	1.743	putative phage protein
yfdr1	2.191	2.945	unknown
UTI89_C2674	2.191	3.653	unknown
roi	2.2	2.988	DNA-binding protein
UTI89_C2681	2.236	2.501	unknown
UTI89_C4883	2.239	4.363	unknown
UTI89_C2663	2.246	1.846	bacteriophage ST64T anti-terminator gp23
UTI89_C2652	2.263	1.947	gene 8-like phage scaffold protein
fmlD	2.275	1.891	fimbrial fimH mannose-binding
ninB1	2.279	2.981	NinB protein encoded within prophage
UTI89_C2673	2.301	2.895	bacteriophage replication protein O
umuD	**2.307**	**5.395**	**DNA polymerase V subunit**
UTI89_C2679	2.309	2.898	phage regulatory protein N
UTI89_C2645	2.322	2.858	gene 7-like, putative phage protein
UTI89_C2650	2.342	3.263	unknown
UTI89_C1186	**2.347**	**4.136**	**DinI**
UTI89_C1282	2.366	2.066	lambda ant-restriction protein
UTI89_C2661	2.397	2.792	gene S-like, putative phage holin
kil2	2.403	2.021	kil protein of bacteriophage HK97
UTI89_C2685	2.456	1.98	putative phage protein
UTI89_C2693	2.474	2.163	unknown
ninB2	2.491	2.862	putative phage NinB protein
rus	2.53	2.868	Holliday-junction resolvase
hkaJ	2.546	2.29	anti-RecBCD 2, putative phage protein
UTI89_C2682	2.546	2.385	putative lambda CIII
ninH	2.563	2.663	putative phage NinH protein
ninE2	2.575	2.36	bacteriophage lambda nin 60-like protein
UTI89_C2687	2.579	2.085	bacteriophage HK97 gp40
UTI89_C2668,	2.594	3.964	unknown
erf	2.631	2.258	bacteriophage HK97 gp40
UTI89_C5143	**2.786**	**7.032**	**putative DinI**
UTI89_C2684	2.888	2.267	bacteriophage kil protein
recN	**2.949**	**15.096**	**recombination and repair protein**

Genes indicated in bold are putative SOS regulon-associated elements.

Table 3.4 Significant changes in differentially expressed transcripts - *S. saprophyticus* 15305 control versus 1/4MIC ciprofloxacin (short-term exposure)

Gene	Fold Change (log_2)	Median Effect Size	Functional Annotation
SSP0843	-0.47	-1.969	amidohydrolase
SSP1254	-0.431	-2.204	Fur-family transcriptional regulator
SSP2255	-0.369	-3.692	50S ribosomal protein L25 Ctc
SSP0308	-0.349	-1.977	unknown
SSP0988	-0.309	-2.205	mannosyl-protein endo-beta-N-acetylglucosamidase
SSP0831	-0.29	-1.76	putative mutS DNA mismatch repair protein
SSP2065	-0.231	-1.626	lipase
SSP1669	-0.222	-1.684	unknown
SSP2275	-0.205	-3.166	Orn Lys Arg decarboxylase
rho	-0.171	-1.582	transcription termination factor Rho
trmD	-0.167	-2.157	tRNA (guanine-N(1)-)-methyltransferase
SSP1294	-0.161	-3.773	penicillin-binding protein 2
SSP1850	-0.134	-2.178	lipoyl synthase
SSP1520	0.141	1.59	succinyl-CoA synthetase subunit alpha
groEL	0.192	1.795	chaperonin
SSP2237	0.192	1.873	pyridoxal biosynthesis lyase PdxS
SSP1797	0.21	2.094	3-oxoacyl-[acyl-carrier-protein] synthase II
SSP1024	0.215	1.861	putative general stress protein
SSP0325	0.225	2.619	acetyl-CoA acetyltransferase
SSP1233	0.229	1.692	geranylgeranyl pyrophosphate synthase
gmk	0.235	2.473	guanylate kinase
SSP1618	0.252	2.29	6-phospho-3-hexuloisomerase domain, PHI
SSP1782	0.266	2.265	hemoglobin-like protein
SSP2198	0.271	2.2	3-hydroxybutyrate dehydrogenase
SSP1593	0.278	4.602	cell division inhibitor protein MraZ
SSP0336	0.28	2.475	glucose-specific PTS transporter IIABC
SSP0333	0.282	1.929	pyruvate oxidase
aspS	0.286	2.449	aspartyl-tRNA synthetase
SSP0052	0.293	5.154	restriction endonuclease S subunit
SSP0499	0.297	2.823	formate nitrite family transporter
SSP1470	0.31	3.996	unknown
SSPP216	0.31	2.033	putative cadmium resistance transporter
capH	0.311	4.685	glycosyl transferase
rplW	0.316	2.015	50S ribosomal protein L23
SSPP115	0.323	2.099	arsenic efflux pump protein
rplF	0.334	1.903	50S ribosomal protein L6
SSP1466	0.352	1.72	thiamine-precursor transporter protein
SSP0960	0.353	1.835	putative gas vesicle protein
SSP1059	0.362	1.586	Xaa-Pro dipeptidase
SSP2075	0.363	1.663	nucleoside transporter
rplE	0.375	1.787	50S ribosomal protein L5
SSP1180	0.377	3.269	16S ribosomal RNA methyltransferase RsmE
SSP0717	0.399	1.812	putative hemolysin III homolog
rpmD	0.404	7.223	50S ribosomal protein L30
SSP2208	0.427	1.508	elongation factor G
SSP1416	0.445	1.516	transketolase
valS	0.461	1.702	valyl-tRNA synthetase

Table 3.4 (continued)

Gene	Fold Change (log_2)	Median Effect Size	Functional Annotation
capL	0.467	1.874	capsular polysaccharide synthesis protein
SSP1124	0.488	2.209	adenine phosphoribosyltransferase
SSP0532	0.503	1.554	major facilitator superfamily permease
SSP0508	0.506	1.844	proton sodium-glutamate symport protein
SSP1005	0.513	2.051	major facilitator superfamily permease
SSP2277	0.527	2.023	unknown
SSP2178	0.535	2.218	ribulokinase
SSP2259	0.543	2.785	translation initiation inhibitor
SSP2346	0.609	3.203	unknown
SSP0197	0.689	4.328	unknown
SSP0296	0.696	2.437	copper chaperone
SSP0750	0.753	2.207	putative phosphopentomutase
recA	**0.765**	**1.82**	**recombinase A**
SSP2138	0.92	1.997	unknown

Genes indicated in bold are putative SOS regulon-associated elements.

Table 3.5 Significant changes in differentially expressed transcripts - *S. saprophyticus* 15305 control versus 1/4MIC ciprofloxacin (long-term exposure)

Gene	Fold Change (log_2)	Median Effect Size	Functional Annotation
SSP0584	-0.501	-1.777	unknown
SSP1390	-0.48	-1.647	methionine sulfoxide reductase regulator MsrR
SSP0895	-0.467	-1.839	unknown
SSP1043	-0.432	-1.935	spermidine acetyltransferase
SSP2255	-0.349	-9.872	50S ribosomal protein L25 Ctc
SSP0485	-0.337	-1.968	major facilitator superfamily permease
SSPP138	-0.315	-2.058	IS431 transposase
SSP0021	-0.277	-2.125	two component response regulator OmpR-like protein
SSP1267	-0.22	-2.108	thioredoxin reductase
clpX	-0.163	-2.868	ATP-dependent protease ATP-binding subunit ClpX
SSP2206	-0.162	-2.167	peptidase
SSP1850	-0.15	-2.705	lipoyl synthase
SSP2074	-0.148	-2.187	unknown
capB	-0.123	-1.645	polysaccharide synthesis length determinator
SSP0052	0.156	2.334	restriction endonuclease S subunit
mutL	0.165	1.641	DNA mismatch repair protein
SSP0623	0.17	1.525	iron-siderophore ABC transporter periplasmic protein
SSP1520	0.188	5.695	succinyl-CoA synthetase subunit alpha
SSP1819	0.191	3.238	NADH:flavin oxidoreductase
prfC	0.192	1.992	peptide chain release factor 3
SSP1300	0.195	2.844	permease
SSP1360	0.243	1.556	putative virulence factor B
rpmD	0.246	1.901	50S ribosomal protein L30
capH	0.248	2.519	glycosyl transferase
SSP1618	0.271	4.58	6-phospho-3-hexuloisomerase domain, PHI
SSP1953	0.282	1.919	thioredoxin reductase
SSP0834	0.283	2.595	sulfur transporter
gatB	0.293	1.579	aspartyl/glutamyl-tRNA amidotransferase subunit B
aroB	0.3	2.241	3-dehydroquinate synthase
SSP1593	0.301	2.943	cell division inhibitor protein MraZ
SSP0499	0.304	2.53	formate nitrite family transporter
SSP0336	0.308	2.463	glucose-specific PTS transporter IIABC
SSP1064	0.308	3.309	DNA polymerase III alpha chain
SSP0948	0.315	2	proton glutamate symporter
dnaK	0.316	1.829	molecular chaperone heat shock protein 70
SSP1175	0.329	3.893	heat-inducible transcriptional repressor
SSP0025	0.33	3.233	beta-lactamase metal-dependent hydrolase
SSP2232	0.34	2.819	ATP:guanido phosphotransferase
SSP1068	0.348	2.358	6-phosphofructokinase
SSP0845	0.351	3.597	unknown
SSP1466	0.361	3.088	putative thiamine-precursor transporter protein
SSP0762	0.368	1.506	aldehyde dehydrogenase
SSP0269	0.375	2.264	oligopeptide-peptide ABC transporter permease
SSP0851	0.375	2.294	acetyltransferase
SSP2242	0.39	2.591	dihydropteroate synthase
rpsM	0.394	3.536	30S ribosomal protein S13
SSP0776	0.402	2.505	ATP synthase F0F1 subunit C

Table 3.5 (continued)

Gene	Fold Change (log_2)	Median Effect Size	Functional Annotation
SSP1358	0.416	2.286	aldo keto reductase
SSP1814	0.418	2.248	argininosuccinate synthase
SSP1176	0.421	1.576	heat shock protein GrpE
SSP0767	0.429	1.836	rRNA or tRNA methylase
SSP1005	0.43	2.005	major facilitator superfamily permease
SSP1729	0.457	1.58	putative bacterial Ig-like 3-domain protein
SSP1627	0.468	1.989	short-chain alcohol dehydrogenase
SSP0208	0.471	4.741	nitroreductase
SSP0960	0.473	2.961	putative gas vesicle protein
SSPP218	0.532	3.139	cadmium efflux system accessory protein
SSP1178	0.538	2.667	chaperone protein DnaJ
SSP0178	0.561	1.559	glycerate kinase
SSP2233	0.593	3.447	McsA modulator of heat shock repressor CtsR
SSP2178	0.662	3.863	ribulokinase
SSP0197	0.676	2.829	unknown
gidB	0.691	2.35	glucose inhibited division protein B
SSP1800	0.77	2.841	putative ATP-depended chaperone ClpB
SSP2138	0.795	1.633	unknown
SSP0296	0.85	3.989	copper chaperone
SSP0769	1.084	1.816	exopolysaccharide-associated tyrosine-phosphatase

Genes indicated in bold are putative SOS regulon-associated elements.

3.2 DEMONSTRATING THE INFLUENCE OF SUB-INHIBITORY CIPROFLOXACIN ON UROPATHOGENENESIS IN A MURINE MODEL OF UTI

Having demonstrated antibiotic-dependent changes *in vitro*, the next logical step was to establish the potential clinical relevance and therapeutic implications *in vivo*. This work used a well defined murine model of UTI and was conducted in the laboratory of Dr. Scott Hultgren (Washington University School of Medicine in St Louis, MO) over four months, with funding by a Canadian Institutes of Health Research - Michael Smith Foreign Study Supplement.

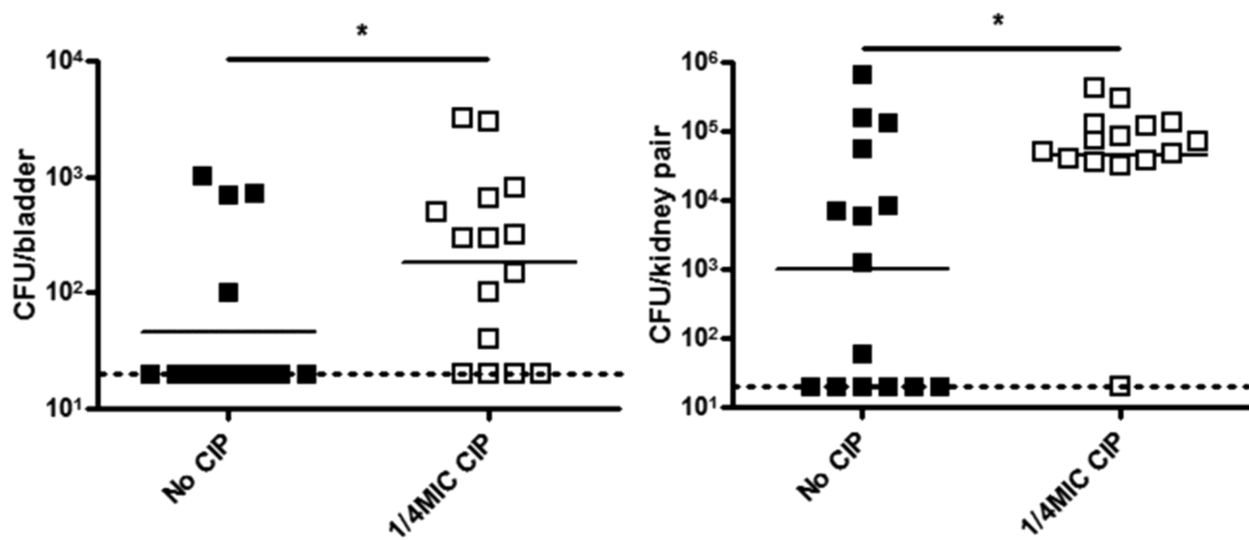
3.2.1 Evaluating the pathogenic potential of ciprofloxacin-primed *S. saprophyticus* and *E. coli* during acute phase infection

The outcome of UTI is generally accepted to be determined within the first 24 hours of inoculation, with underlying host and pathogen factors contributing to either resolution, or establishment of chronic infection. Generally, these trends are observed at this time as a bimodal distribution of bacterial load, with ~50% of C3H/HeN mice revealing signs of resolution, and the remainder sustaining high titers. The bacterial load after initial infection with untreated or ciprofloxacin-primed uropathogens was assessed to determine the effect of sub-inhibitory antibiotics on colonization after 24 hpi. A typical bimodal distribution of bacterial loads was observed at this time point, with ciprofloxacin-priming prior to infection resulting in higher overall bladder and kidney titers for both *S. saprophyticus* 15305 and *E. coli* UTI89 after 24 hours (Figure. 3.15). Almost 100% of murine kidneys showed no signs of infection clearance at this time point for both organisms primed with ciprofloxacin.

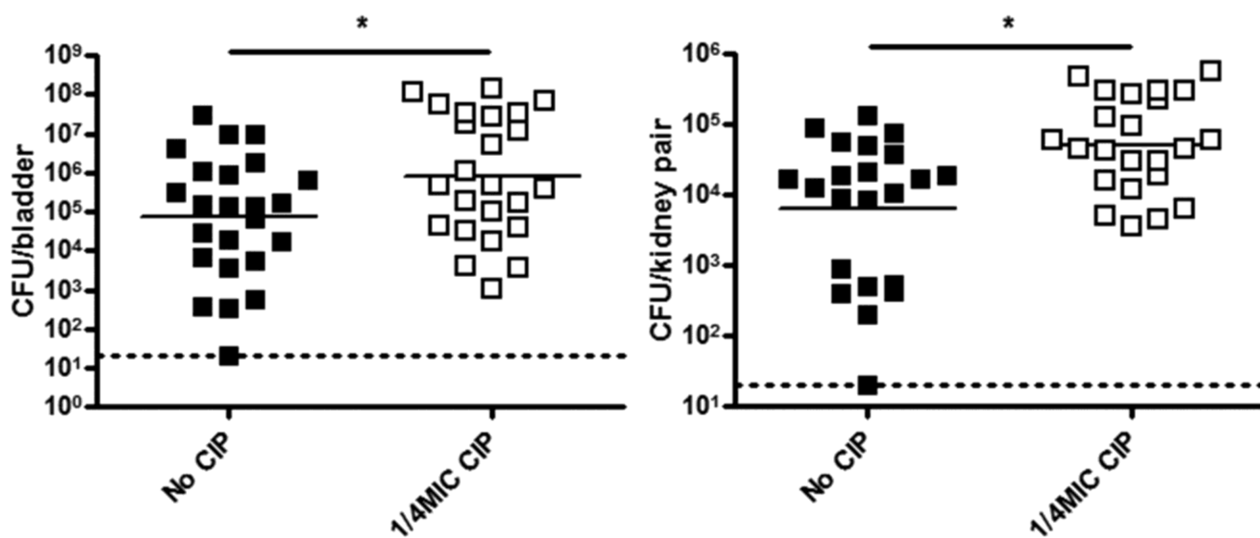
Figure 3.15. *S. saprophyticus* 15305 and *E. coli* UTI89 titers in C3H/HeN mouse bladders and kidneys following 24 hour infections. Starter cultures were reconstituted in fresh media supplemented with 1/4MIC ciprofloxacin for 4 hours prior to infection. Dotted line indicates limit of detection. Means from at least three independent experiments shown with significance. Significance was determined using Mann-Whitney test (Gaussian approximation) (*, $P < 0.05$).

Figure 3.15

S. saprophyticus 15305



E. coli UTI89



3.2.2 Establishing the effect of ciprofloxacin priming on chronic UTI risk in *S. saprophyticus* and *E. coli* infected mice

In order to determine the frequency of chronic cystitis and pyelonephritis in antibiotic-primed versus control pathogens, infection periods were extended to 14 days post-inoculation (dpi). C3H/HeN mice were either inoculated with untreated or ciprofloxacin-primed *S. saprophyticus* 15305 and *E. coli* UTI89 as described. Following sacrifice, bladders and kidneys were plated, and uropathogens enumerated to determine bacterial load. In addition to increasing bacterial load 24 hpi, ciprofloxacin-priming resulted in significantly worse infections 14 dpi ($P < 0.05$) (Figure 3.16). The clinical threshold for assessing lifetime chronic infection risk is the presence of *E. coli* UTI89 loads of $>10^4$ cfu/mL at 14 dpi (such data does not exist for *S. saprophyticus*) (Hannan *et al.*, 2010). Control animals infected with *E. coli* UTI89 demonstrated expected bimodal results, with 40% of mice spontaneously resolving their infections after 14 days. Conversely, ciprofloxacin-priming resulted in 100% of mice developing chronic *E. coli* UTI89 infections. Similarly, 50% of control mice showed signs of kidney clearance while only 20% showed similar results for ciprofloxacin-primed groups. Although there are no established clinically significant *S. saprophyticus* 15305 infection thresholds for this model, it is interesting that 100% of mice resolved cystitis in the control group while 50% maintained bladder titers with ciprofloxacin-priming. In addition, 100% of primed *S. saprophyticus* 15305 treated mice maintained at least some infectious kidney titer, while only 60% exhibited the same result in control groups.

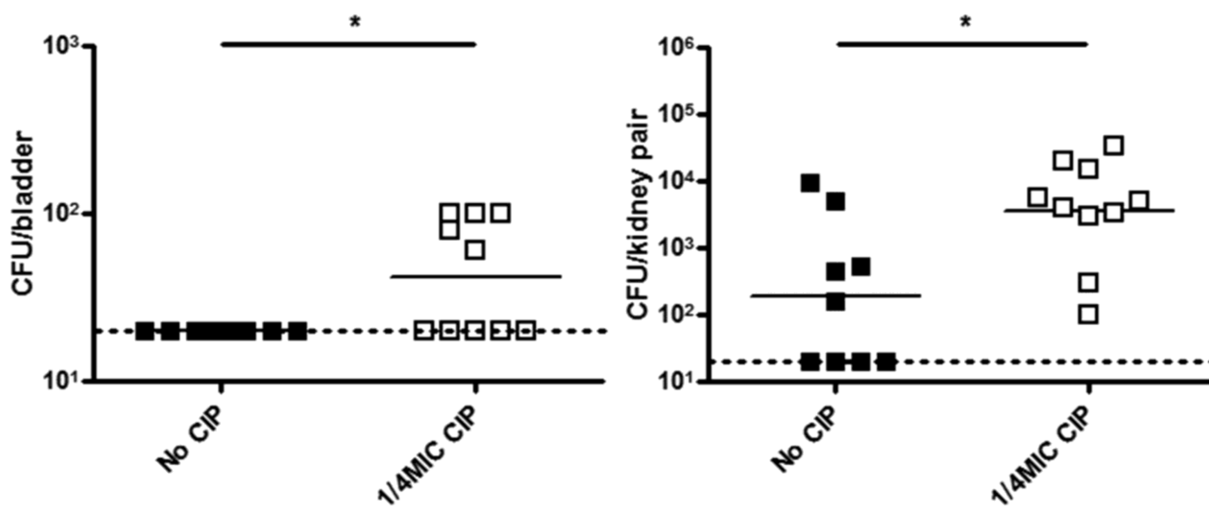
3.2.3 Evaluating ciprofloxacin modulation of pathogenic mechanisms which contribute to chronic infection

Changes in intracellular bladder populations during the early stages of infection were assessed using gentamicin-protection assays (3.5 hpi) for *S. saprophyticus* 15305, and microscopy (6 hpi) for *E. coli* UTI89. Analysis parameters varied as both *E. coli* UTI89 and *S. saprophyticus* 15305 exploit distinct pathogenic mechanisms to establish chronic infection. *E. coli* IBC formation was detected by staining for *lacZ* expression using X-Gal. IBCs are visible as punctate blue stains under examination using a dissecting

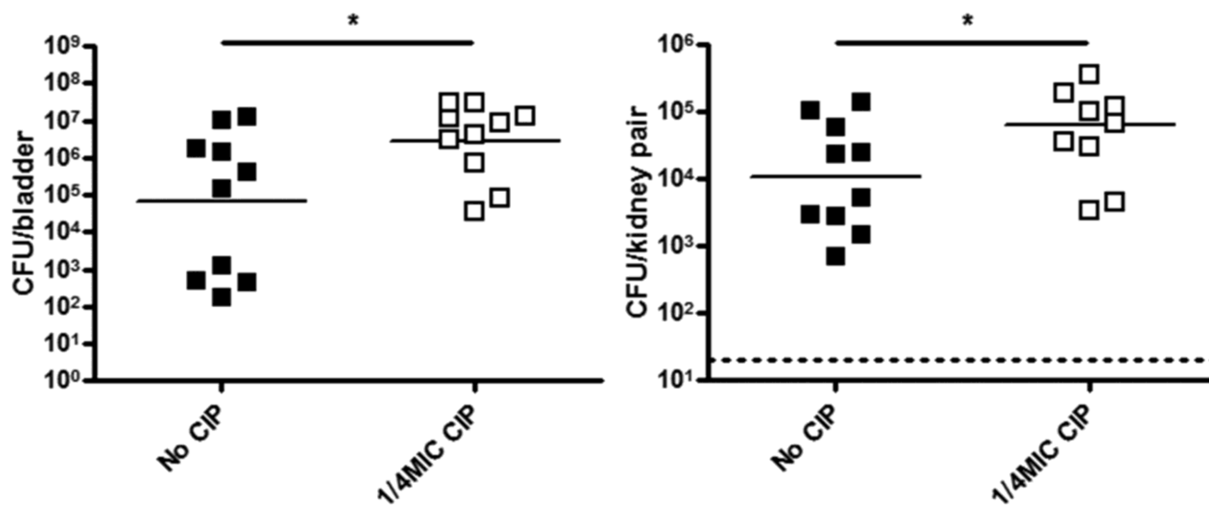
Figure 3.16. *S. saprophyticus* 15305 and *E. coli* UTI89 titers in C3H/HeN mouse bladders and kidneys following 14 day infections. Starter cultures were reconstituted in fresh media supplemented with 1/4MIC ciprofloxacin for 4 hours prior to infection. Dotted line indicates limit of detection. Means from at least three independent experiments shown with significance. Significance was determined using Mann-Whitney test (Gaussian approximation) (*, $P < 0.05$).

Figure 3.16

S. saprophyticus 15305



E. coli UTI89



microscope (Figure 3.17A). This technique indicated significantly more IBCs in ciprofloxacin-primed groups versus control ($P < 0.01$) (Figure 3.17B). *Ex vivo* gentamicin protection assays of murine bladders revealed a similar trend, with categorical increases in intracellular *S. saprophyticus* 15305 populations observed in ciprofloxacin-primed organisms ($P = 0.0019$) (Figure 3.17C). Although total invasion events were rare in *S. saprophyticus* 15305, intracellular reservoirs were comparatively much lower in the untreated group, with only 30% of mice demonstrating pathogen presence compared to 100% in treated groups.

3.2.4 Investigating IBC and cellular morphology in ciprofloxacin-primed *E. coli*

In addition to being more numerous, IBCs also appeared larger under light microscopy following antibiotic exposure. This observation was further explored using a green fluorescent protein (GFP) expressing *E. coli* UTI89 strain and confocal microscopy. Murine bladders were removed and prepared as previously described, but fixed and stained with fluorescent wheat germ agglutinin (WGA) and SYTO9 for cell surface and nuclei detection respectively. Appearance of control *E. coli* UTI89 was unremarkable as IBCs demonstrated characteristic tight, globular clustering (Figure 3.18A). Examination of ciprofloxacin-primed *E. coli* UTI89 revealed larger, diffuse IBCs with atypical morphology (Figure 3.18B). Early filamentation was noted in several of the IBCs belonging to the ciprofloxacin treated *E. coli* UTI89 group (Figure 3.18C). Volumetric analysis of the IBCs confirmed that ciprofloxacin-priming significantly increased overall size 2.0-fold ($P = 0.0007$).

The ability for ciprofloxacin to induce filamentation was investigated further as this process might contribute to observed early IBC evacuation. Cultures of *E. coli* UTI89 were treated with sub-inhibitory ciprofloxacin and imaged using TEM. Control *E. coli* UTI89 were unremarkable, appearing ~1-2 µm in length (Figure 3.19A). Most organisms in the ciprofloxacin treatment group appeared similar in nature; however, ~10% of the overall population presented with the observed filamenting phenotype, sometimes increasing to >10 µm in length (Figure 3.19B).

Figure 3.17. *E. coli* UTI89 IBC detection using lacZ staining in C3H/HeN mouse bladders (A) and quantification (B) following 6 hour infections. Number of intracellular *S. saprophyticus* 15305 isolated from murine bladders following a gentamicin protection assay (C). Whole bladders were plated and *S. saprophyticus* colonies identified using lacZ staining. Means from at least two independent experiments are shown with significance. Significance determined using Mann-Whitney test (B) with Gaussian approximation (C) (**, $P < 0.01$; ***, $P = 0.0019$).

Figure 3.17

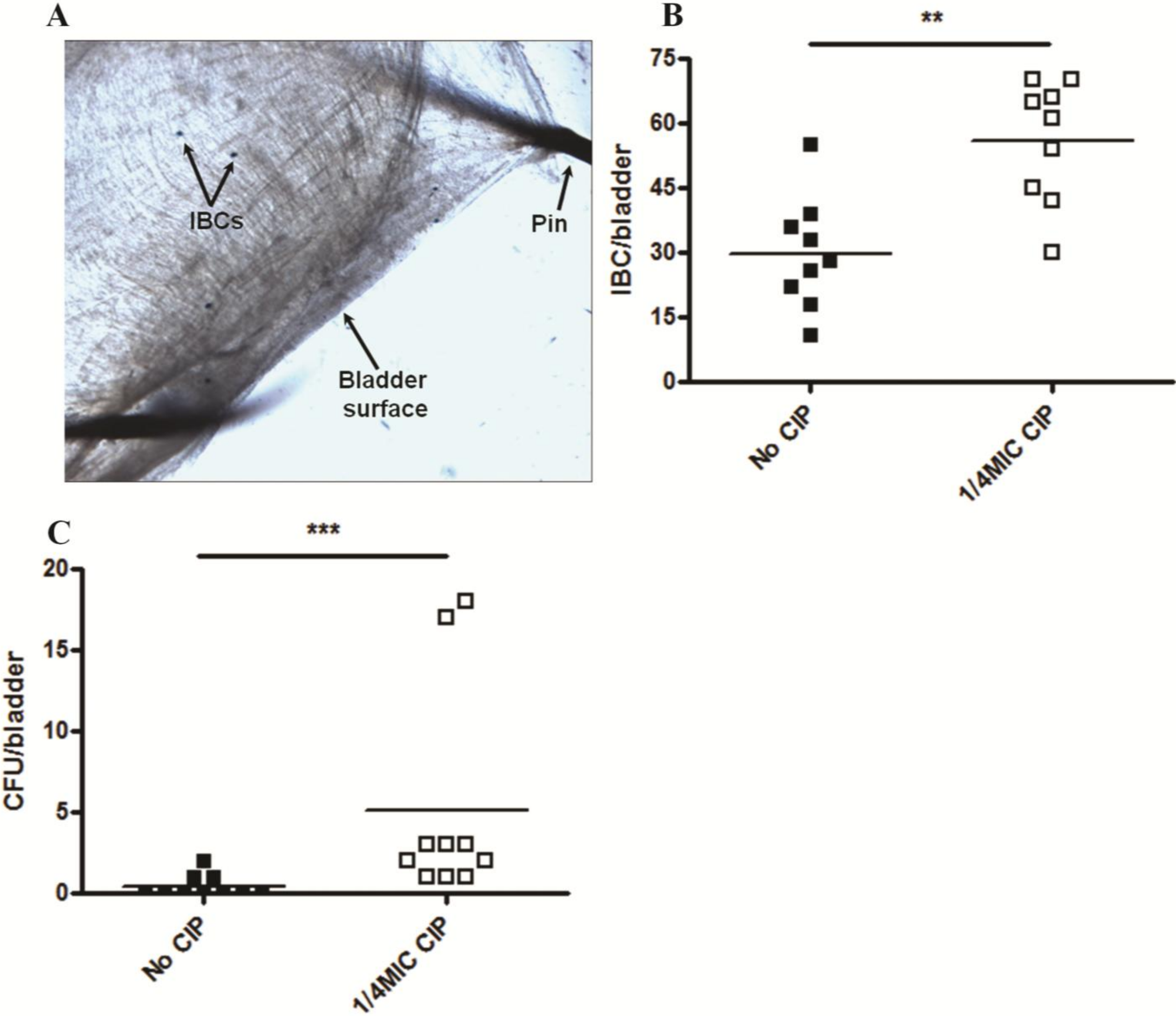


Figure 3.18. Representative confocal images of control (A) and antibiotic pre-treated (B) *E. coli* UTI89 IBCs in C3H/HeN mouse bladders following 6 hour infections (red = GFP/UTI89; blue = SYTO9/nuclei; green = WGA/cell). Early pathogen evacuation from an IBC via filamentation in a ciprofloxacin pre-treated sample is shown (indicated with arrows - C). Volumetric analysis of confocal images is depicted for both control and antibiotic pre-treated IBCs (D). Means from at least two independent experiments are shown with significance. Significance was determined using a Mann-Whitney test (***, $P = 0.0007$).

Figure 3.18

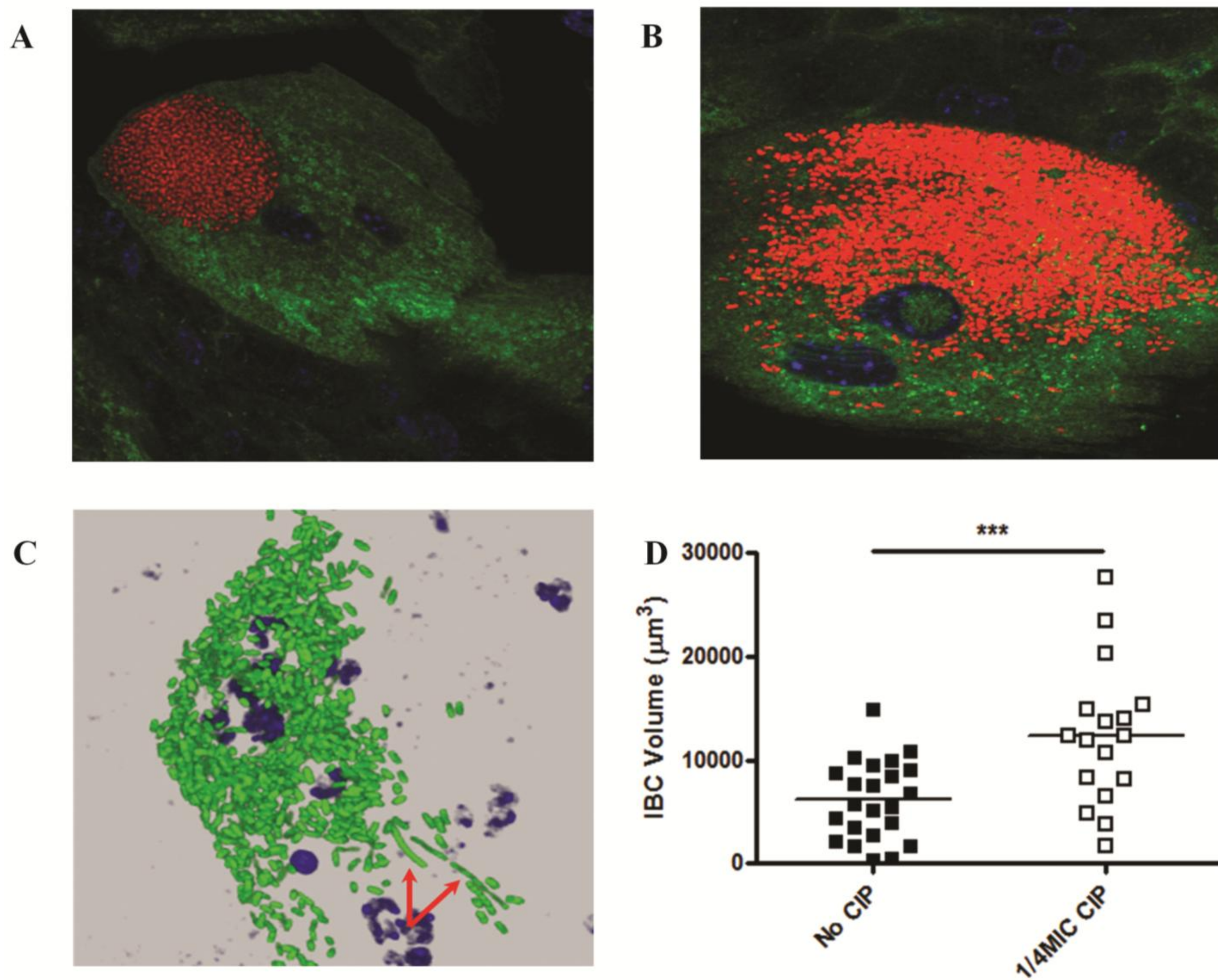
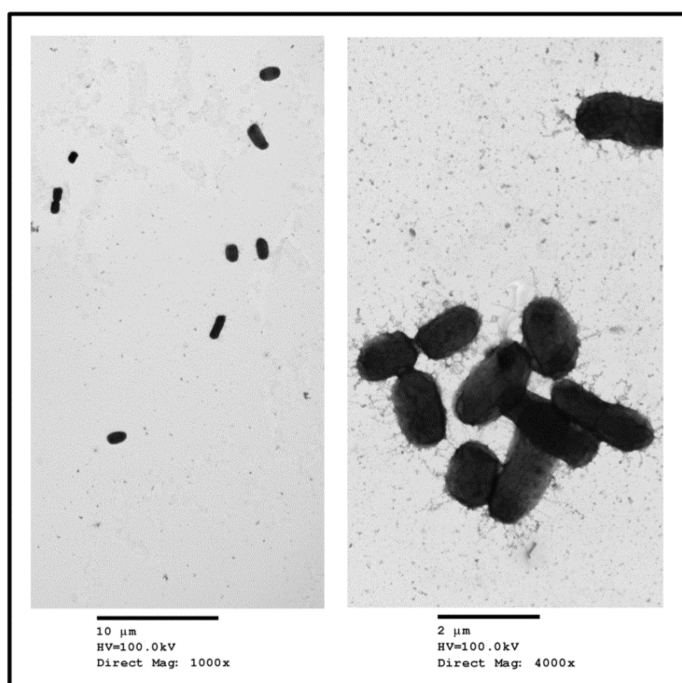


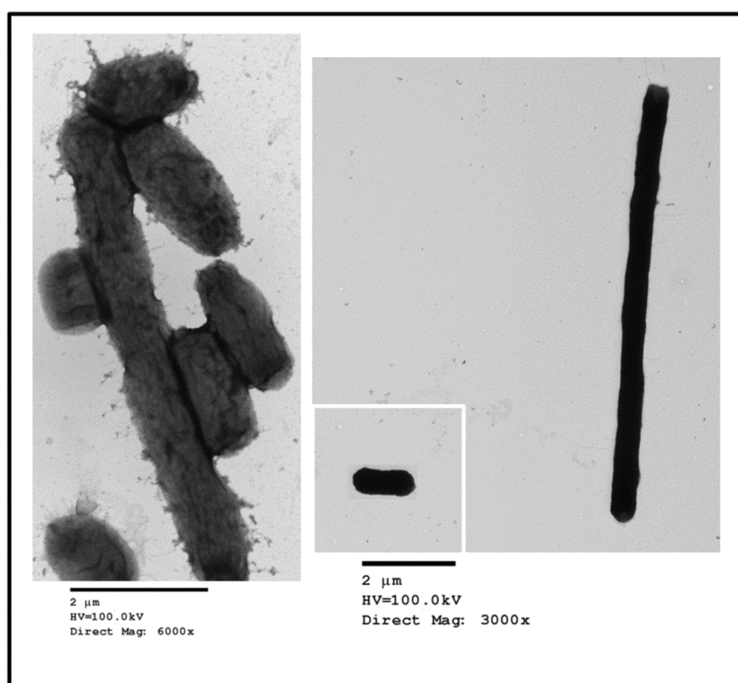
Figure 3.19. Representative TEM images of non-treated (A) and 1/4MIC ciprofloxacin treated (B) *E. coli* UTI89 filamentation. The first panel depicts individual and clustered rod and coccoidal *E. coli* while the second panel reveals filamenting and non-filamenting organisms. Magnification for each micrograph is indicated.

Figure 3.19

A



B



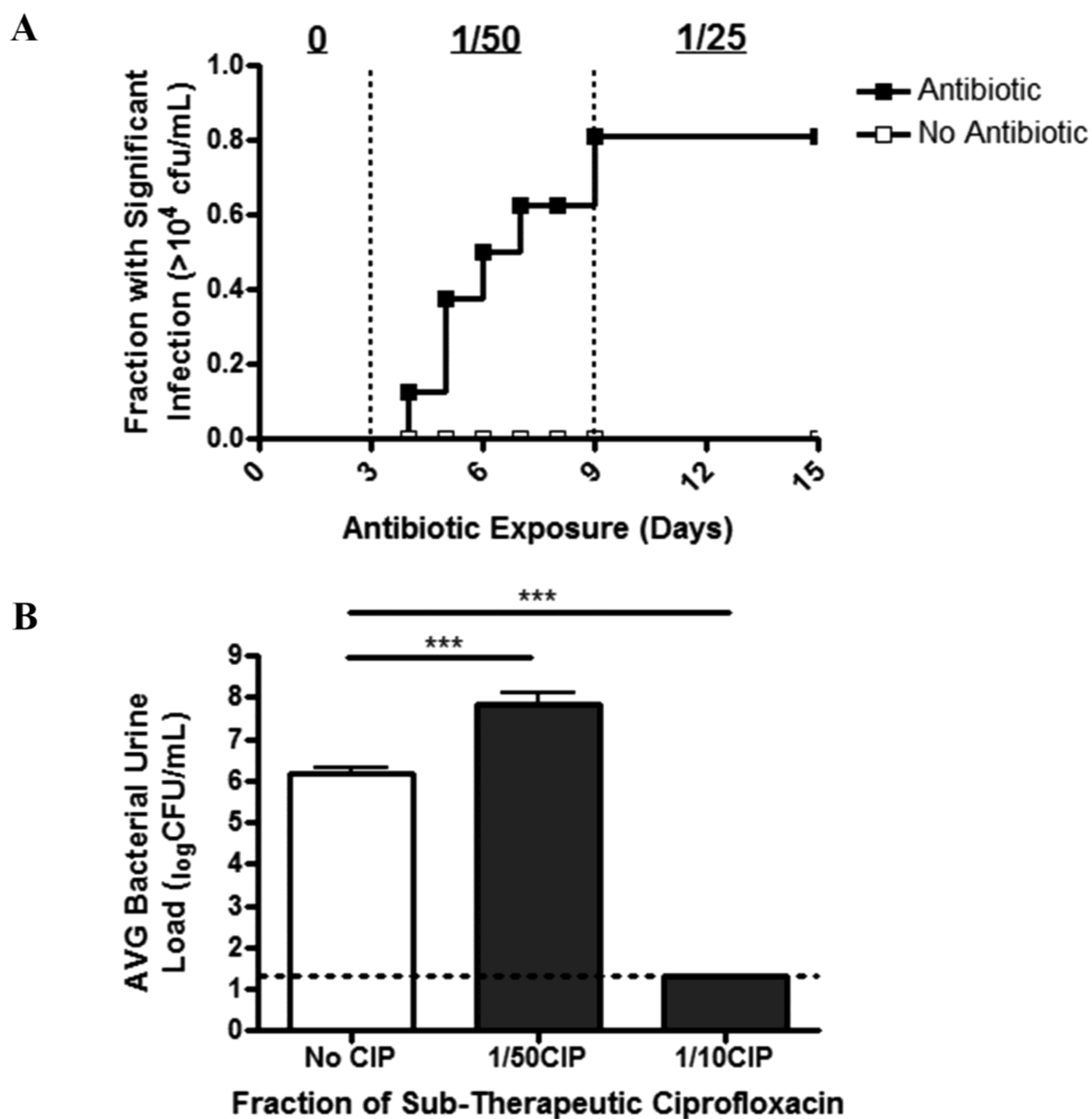
3.2.5 Assessing the effect of sub-therapeutic ciprofloxacin on recurrence risk in 'resolved' mice and infection severity chronically infected mice

Thus far, experimental models have only made use of ciprofloxacin-primed uropathogens, with mice receiving no antibiotic exposure. To overcome this, a new model of infection was established whereby mice were supplemented with sub-therapeutic ciprofloxacin in their drinking water daily. Sub-therapeutic antibiotic-dependent changes in bacterial urine titers of mice who were previously inoculated with *E. coli* UTI89 but had spontaneously resolved ($<10^4$ cfu/mL bacterial urine load) their infection were initially considered. Urine titers were tracked for three days in the absence of antibiotic to determine baseline cfu/mL. Over this period no mice presented with clinically significant infection ($>10^4$ cfu/mL) (Figure 3.20A). Antibiotics at 1/50th the empirical therapeutic concentration were then supplemented into the drinking water and changes in bacterial urine load were tracked. This dose significantly increased the frequency of mice with clinically significant infection from 0% to 80% occurrence (P = 0.0131) (Figure 3.20A). The dose was further increased to 1/25th the empirical therapeutic dose with no changes observed in the remaining mice (Figure 3.20A). Increasing the dose to 1/10th the empirical therapeutic dose resulted in retraction of bacterial urine loads to the limit of detection, suggesting that observed antibiotic-dependent increases were not due to the appearance of resistant mutants (data not shown). Although it can be concluded that ciprofloxacin initiated infection recurrence, it is unclear whether this was due to re-infection with a new strain or recalcitrance of the previously inoculated pathogen.

The effect of inadequate dosing was also assessed in mice with chronic infection ($>10^4$ cfu/mL for ≥ 14 dpi). This experiment was undertaken to test if antibiotics would worsen an already established infection, such as might occur during low-dose, extended use prophylaxis. Chronically infected mice were supplemented with 1/50th the empirical therapeutic dose for three days, with urine titers measured after this period. This significantly increased *E. coli* UTI89 urine load by an order of magnitude of 1.7 (P < 0.001) (Figure 3.20B). Again, treatment with 1/10th of the therapeutic dose was sufficient to decrease cfu/mL to the limit of detection indicating spontaneous resistant mutants did

Figure 3.20. Fraction of 'resolved' C3H/HeN mice presenting with clinically significant (>10^4 cfu/mL) bacterial urine titers following sub-therapeutic ciprofloxacin dosing over time (N = 10 mice; A). Antibiotic dosing period is indicated in the upper x-axis as fractions of the therapeutic dose. Urine titers of chronically infected mice receiving either no antibiotic or sub-therapeutic ciprofloxacin for three days (B). Means from at least two independent experiments are shown with significance. Significance was determined using a Logrank (A; $P = 0.0131$) or one-way ANOVA and Bonferroni's multiple comparison test (B) (***, $P < 0.001$).

Figure 3.20



not contribute to increased titers during dosing (Figure 3.20B). The application of ciprofloxacin resulted in more significant infections than when organisms were primed prior to inoculation. This observation suggests that additional factors unrelated to the pathogens themselves are potentially enhancing virulence.

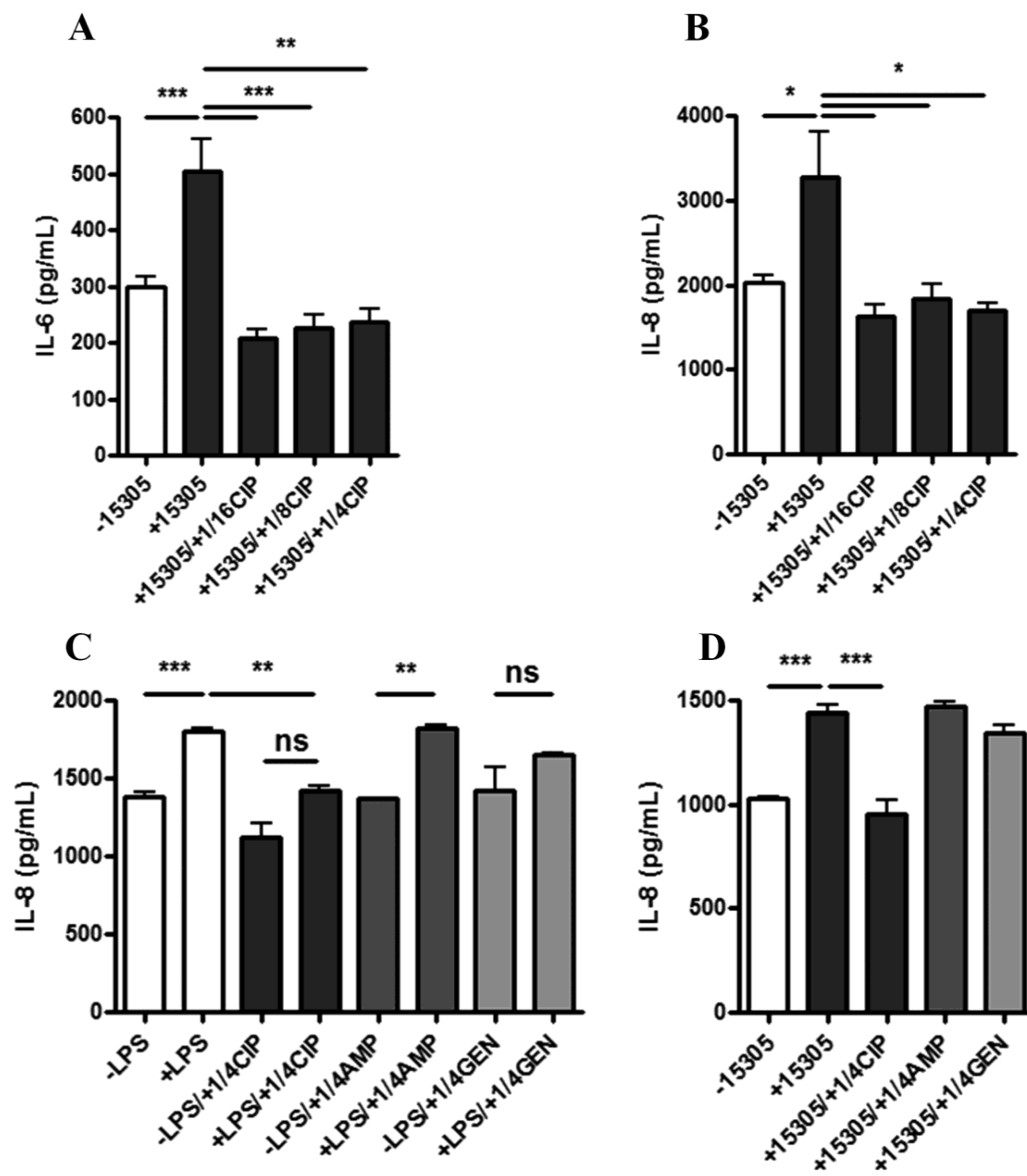
3.2.6 Evaluating the capacity of ciprofloxacin to modulate urothelial immune responses

It was postulated that the changes in infection frequency and severity observed in mice could in part be attributed to the immunomodulatory capacity of ciprofloxacin on the host. This was first tested in a controlled *in vitro* experiment using bladder cell lines. Cytokines interleukin (IL)-6 and IL-8 were analyzed for their role in recruiting neutrophils to urothelial infection sites using a Luminex® bead-based assay. Baseline expression was compared to infection groups in the absence of antibiotics and results showed a typical spike in cytokine secretion for both molecules ($P < 0.001$) (Figure 3.21A, B). All sub-inhibitory ciprofloxacin concentrations reduced IL-6 and IL-8 secretion to below baseline levels ($P < 0.05$; $P < 0.01$; $P < 0.001$). The finding that even low ciprofloxacin doses induced suppression of cytokine release was reflective of the drug's potency at physiologically relevant concentrations.

The findings observed in the T24 bladder cell lines were recapitulated in 5637 bladder cells. Ampicillin and gentamicin were also tested at 1/4MIC levels to assess the specificity of immune suppression. *S. saprophyticus* 15305 infections were carried out as described, and supernatants were analyzed for IL-8 expression using an enzyme linked immunosorbent assay (ELISA). While ciprofloxacin treatment reduced IL-8 secretion to below baseline levels ($P < 0.05$) (Figure 3.21D), ampicillin and gentamicin did not induce this effect. An additional experimental group containing LPS was applied as a cell-free control to ensure *S. saprophyticus* 15305 was not influencing the release of cytokine. LPS in the absence of antibiotic caused a predicted spike in IL-8 ($P < 0.001$) (Figure 3.21C), however, ciprofloxacin depressed IL-8 secretion ($P < 0.01$). Although ampicillin did not influence IL-8 secretion, gentamicin did demonstrate slight but not significant immunomodulatory activity.

Figure 3.21. IL-6 (A) and IL-8 (B) cytokine expression profiles for T24 bladder cells infected with *S. saprophyticus* 15305 exposed to various subinhibitory levels of ciprofloxacin. Release of IL-8 from 5637 bladder cells following treatment with either LPS (C) or *S. saprophyticus* 15305 (D) in the presence of 1/4MIC of either ciprofloxacin (C), ampicillin (A), or gentamicin (G) as indicated. Significance was determined using one-way ANOVA and Dunnett's (A,B) or Bonferroni's (C,D) multiple comparison tests (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns = not significant).

Figure 3.21



3.2.7 Evaluating the immunomodulatory effects of sub-therapeutic ciprofloxacin during early stages of UTI *in vivo*

It was hypothesized that the immunomodulatory effects of ciprofloxacin *in vitro* would suppress the secretion of pro-inflammatory mediators and decrease immune cell infiltrate *in vivo*. Both *E. coli* UTI89 and mock (PBS) infected mice receiving sub-therapeutic ciprofloxacin were found to have significantly fewer PMNs in urine sediments compared to controls ($P < 0.05$; $P < 0.001$) (Figure 3.22). The results for the mock infected, ciprofloxacin treated group were especially noteworthy, with no measurable PMN score being achieved.

The cause of the reduced immune cell infiltrate in ciprofloxacin treated mice was postulated to be related to modulation of cytokine secretion. This was assessed using a Luminex® bead-based multiplex assay of bladder tissue homogenates. A total of seven urinary-relevant cytokines were assessed and included IL-1β, IL-6, KC (IL-8), G-CSF, IL-17, IL-10, and TNFα. All produced reliable profiles with the exception of TNFα (data not shown). Changes in IL-17 secretion were unremarkable in either control or ciprofloxacin treatment groups (Figure 3.23). Similarly, KC was not significantly impacted by ciprofloxacin, although there was a trend of reduced secretion when antibiotic was present (Figure 3.23). All other cytokines were significantly affected by the presence of ciprofloxacin. The pro-inflammatory mediators IL-1β and IL-6 were suppressed while secretion of the anti-inflammatory cytokine IL-10 was increased ($P < 0.05$; $P < 0.01$; $P < 0.001$) (Figure 3.23). These cytokine alterations could reasonably account for the observed decrease in immune cell infiltrate in urine sediments. In contrast, the pro-inflammatory cytokine G-CSF was significantly increased in the presence of ciprofloxacin and *E. coli* UTI89 infection ($P < 0.05$) (Figure 3.23). Of note, *E. coli* have previously been reported to increase the expression of this cytokine. It remains unclear how ciprofloxacin influences this process.

Figure 3.22. PMN counts collected from urine sediments of either *E. coli* UTI89 or mock (PBS) infected mice treated with or without 1/50 sub-therapeutic ciprofloxacin supplemented drinking water (3 day exposure). Means from at least two independent experiments are shown with significance. Significance was determined using one-way ANOVA and Bonferroni's multiple comparison test (*, $P < 0.05$; ***, $P < 0.001$).

Figure 3.22

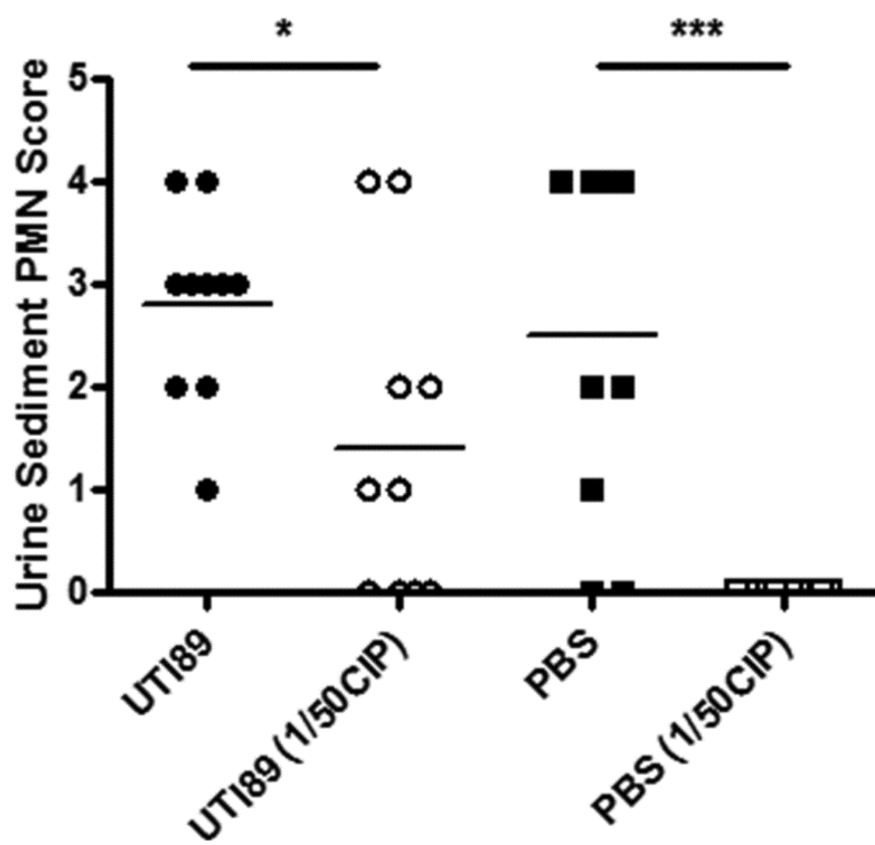
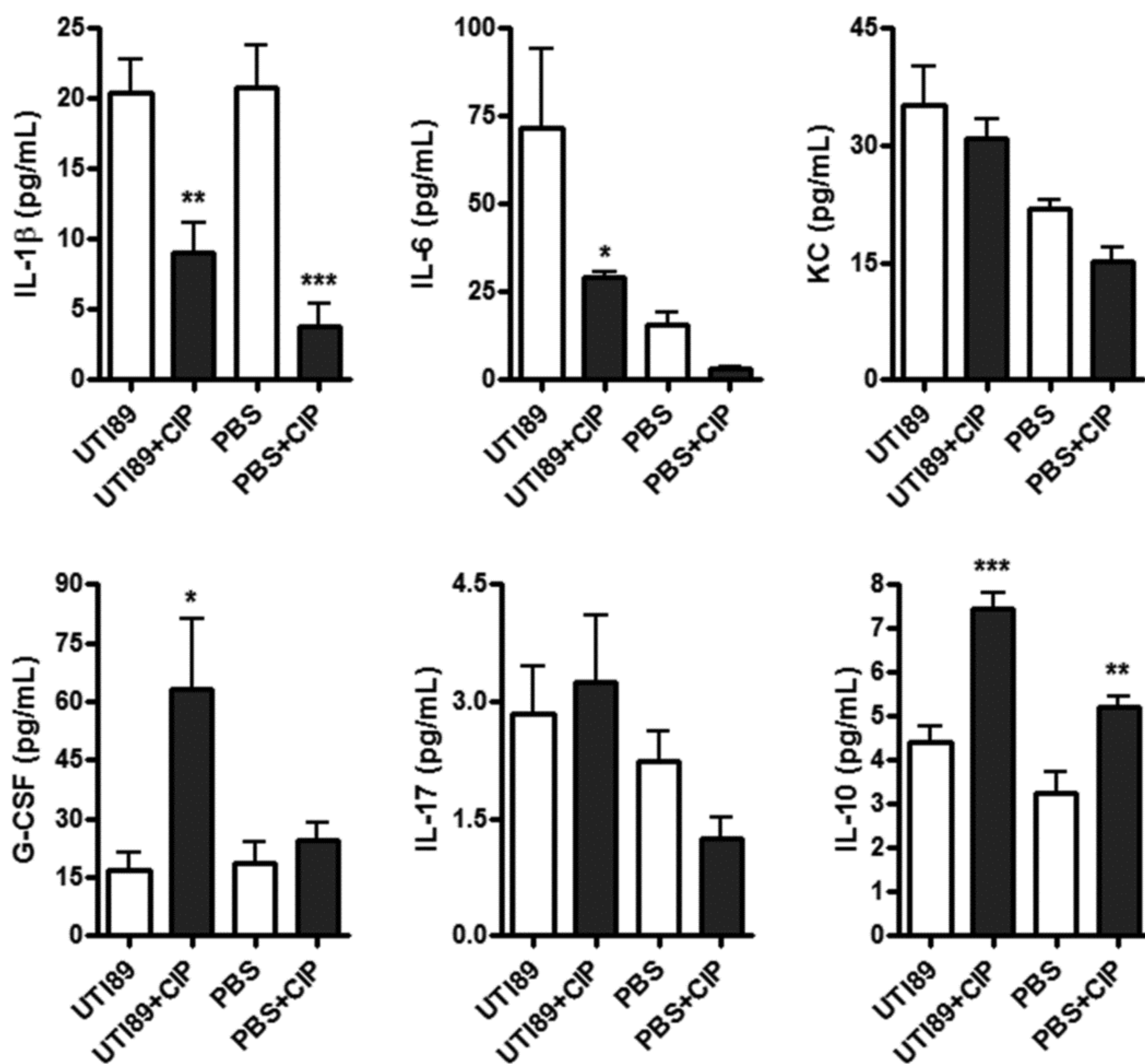


Figure 3.23. Murine bladder cytokine secretion following 3.5 hour *E. coli* UTI89 or mock (PBS) infection in the presence or absence of 1/50 sub-therapeutic ciprofloxacin supplemented drinking water (3 day exposure). Means from at least two independent experiments are shown with significance. Significance was determined using one-way ANOVA and Bonferroni's multiple comparison test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 3.23



3.2.8 Assessing the influence of sub-therapeutic ciprofloxacin on host and pathogen responses *in vivo*

As ciprofloxacin modulates the immune response, it was hypothesized that mice receiving sub-therapeutic ciprofloxacin would develop less severe inflammation and decreased risk of developing chronic infection than control mice. Sub-therapeutic ciprofloxacin treatment did not significantly affect bacterial urine loads compared to untreated controls (Figure 3.24). Still, mice presented with lower infection titers at 14 dpi when they received ciprofloxacin supplemented drinking water (Figure 3.24D, E). Early kidney infection titers were slightly higher in the mice receiving antibiotic, compared to controls (Figure 3.24B). In addition to decreased titer, bladders from ciprofloxacin treated mice weighed significantly less than those from the control group, suggestive of decreased immune infiltration ($P < 0.05$) (Figure 3.24C).

3.2.9 Evaluation of infection severity in a murine ciprofloxacin prophylaxis model of recurrent UTI

The possibility that low-dose prophylaxis promotes virulence and enhances pathogenesis was investigated. Changes in bacterial urine load were assessed at least every two days to track the course of infection using a prophylaxis therapy model. As expected, bacterial urine loads decreased with the application of co-trimoxazole, with the exception of two mice in experiment 2 (Figure 3.25). The following 7 day rest period revealed dynamic changes in *E. coli* UTI89 urine titer, with frequent recurrences observed. In general, prophylactic therapy did not appear to improve the frequency of infection resolution over the 7 day treatment period compared to non-treated controls. Indeed, 10% of mice in the prophylactic group presented with clinically significant infection while 15% did so in the control group at the study's end (Figure 3.25).

Figure 3.24. Bladder and kidney titers of *E. coli* UTI89 inoculated mice receiving 1/50th sub-therapeutic ciprofloxacin supplemented drinking water or control. Mice were sacrificed at either 24 hpi (A, B) or 14 dpi (D, E) to determine infection severity. Bladder weight was also measured at 24 hpi (C). Means from at least two independent experiments are shown with significance. Significance was determined using Mann-Whitney test (Gaussian approximation) (*, $P < 0.05$).

Figure 3.24

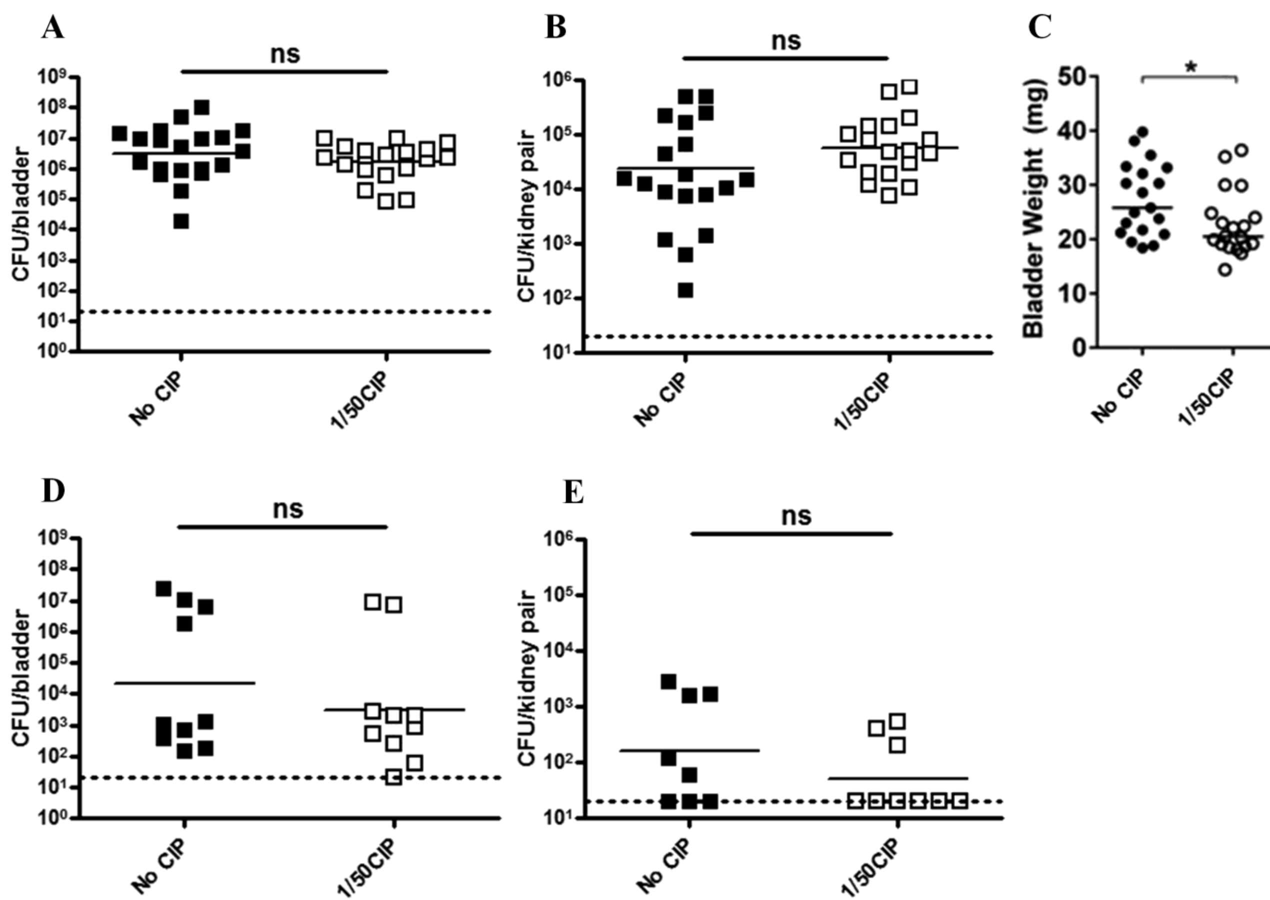
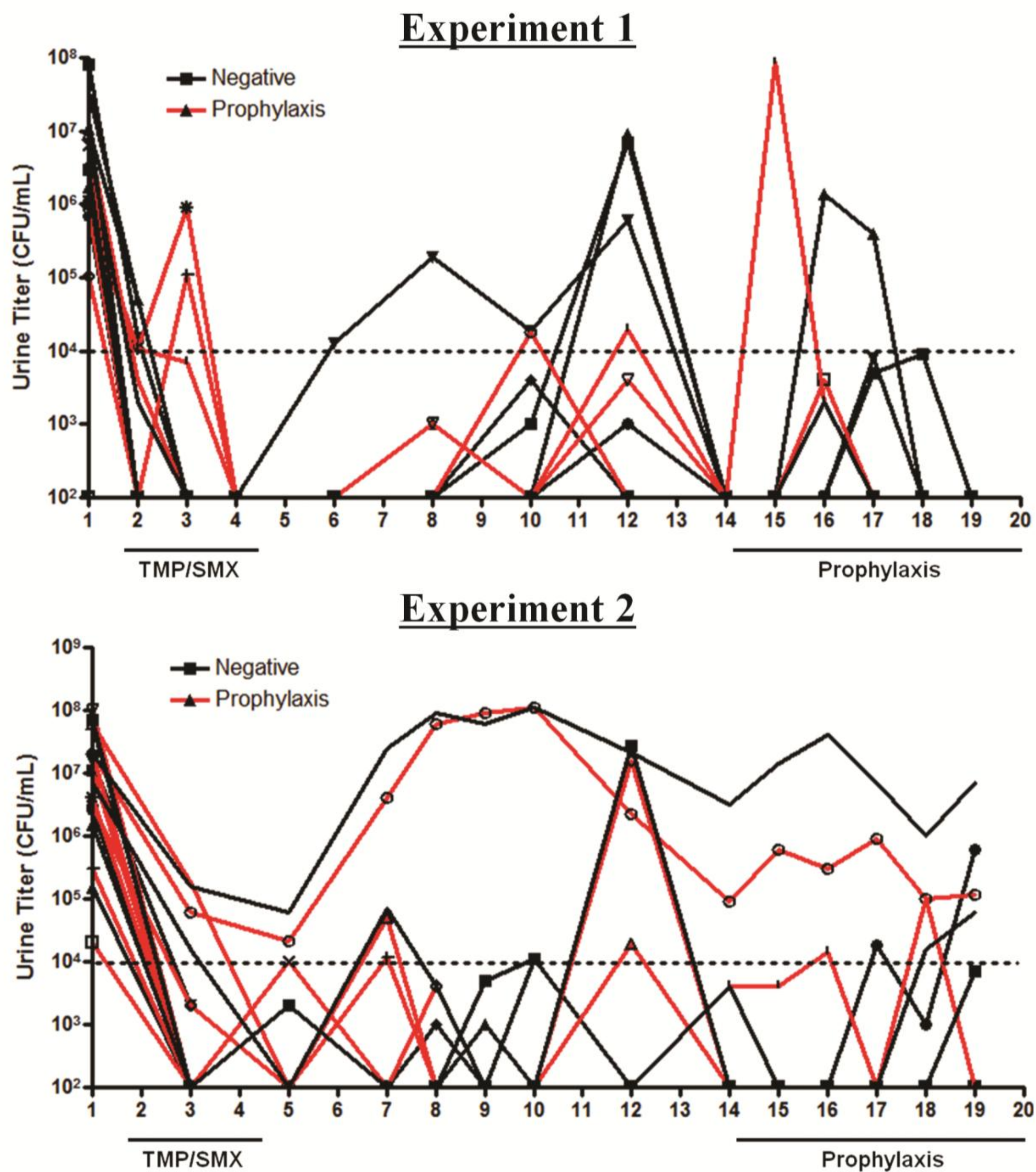


Figure 3.25. Two experimental replicates follow *E. coli* UTI89 urine titers in C57BL/6 mice treated prophylactically with ciprofloxacin (red) or vehicle (water - black) via daily gavage. Each line represents the bacterial urine load for an individual mouse. Axis labels indicate the start of co-trimoxazole (TMP/SMX) therapy and period of prophylaxis dosing. Dotted line indicates the cut off for clinically significant ($>10^4$ cfu/mL) infection.

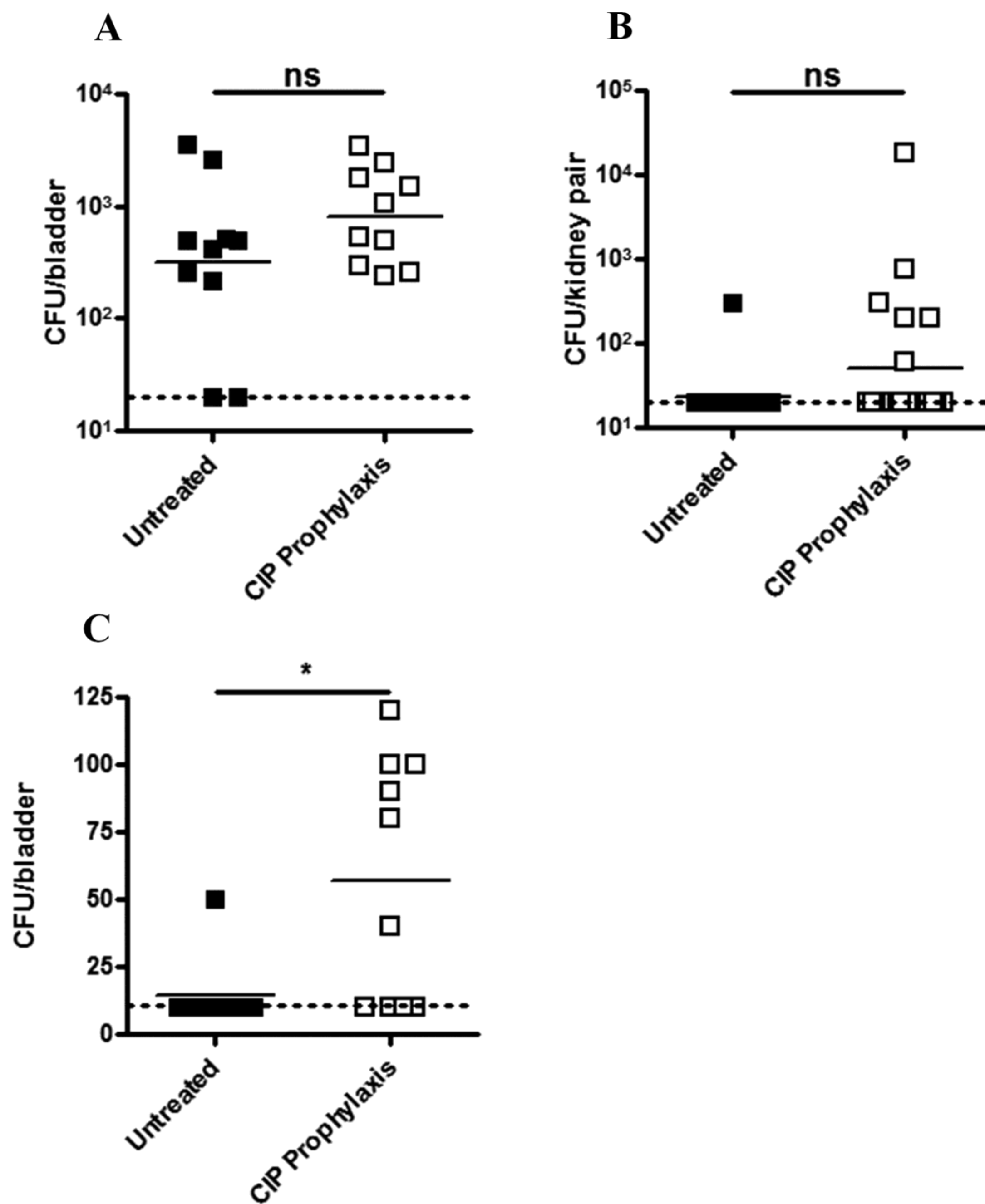
Figure 3.25



The unremarkable urine results from the prophylaxis experiments necessitated further investigation. Therefore, upon sacrifice both bladders and kidneys were evaluated to assess *E. coli* UTI89 colonization. Although both organs demonstrated greater bacterial loads in the prophylaxis group, this difference was not significant (Figure 3.26A, B). However, comparison of *E. coli* UTI89 intracellular bladder reservoirs revealed prophylaxis significantly increased the frequency of urothelial invasion ($P < 0.05$). The inability of prophylaxis to eradicate infection is worthy of further investigation.

Figure 3.26. *E. coli* UTI89 titers in C57BL/6 mouse bladders (A) and kidneys (B) following a 1 week prophylactic treatment regimen with either ciprofloxacin or vehicle (water). Gentamicin protection assays indicate the presence of intracellular *E. coli* reservoirs (C). Dotted line indicates limit of detection. Means from at least two independent experiments are shown with significance. Significance was determined using an unpaired, two-tailed t-test. (ns = not significant; *, $P = 0.0148$).

Figure 3.26



3.3 UNDERSTANDING THE ABILITY OF UROPATHOGENS TO PERSIST DESPITE ADEQUATE ANTIBIOTIC INTERVENTION DURING INFECTION

The inability of antibiotics to eradicate bacteria during chronic diseases such as recurrent UTI is attributed to the presence of MDT persister cells. Yet, despite the susceptibility of *E. coli* UTI89 to ciprofloxacin, titers were unchanged by prophylaxis, and actually increased slightly in some cases (Figure 3.25A, B). As persister cells are known to form in response to environmental stress, it was hypothesized that antibiotics are capable of promoting onset of this MDT state. In designing studies to address this hypothesis, it was deemed important to characterize the nature of uropathogen persisters in order to evaluate novel therapeutic modalities.

Persister cells are believed to benefit from MDT through complete, global metabolic dormancy. Therefore, two hypotheses were considered to evaluate the contribution of dormancy towards persistence: i) dormancy is necessary and sufficient to convey MDT such that persisters will respond to different antibiotics with the same levels of survival; and ii) different antibiotics will induce global dormancy such that surviving persisters will demonstrate tolerance to both lethal levels of the same and different agents (cross-tolerance). This work challenges the notion that persister cells represent truly dormant variants of the overall population.

3.3.1 Different classes of antibiotics induce persister cell formation in both *S. saprophyticus* and *E. coli*

The ubiquity of antibiotic-dependent persister induction was assessed using ciprofloxacin, ampicillin, and gentamicin against both Gram-negative (*E. coli* CFT073) and Gram-positive (*S. saprophyticus* 15305) organisms. The persister traits of *E. coli* CFT073 are better characterized than those of *E. coli* UTI89, justifying its initial use for these experiments. Importantly, growth phase has a significant impact on persister levels, requiring growth rate characterization of all strains utilized prior to investigation. In general, all strains of *E. coli* used throughout this study demonstrated similar growth rates, while *S. saprophyticus* 15305 was comparatively distinct, warranting adjustment of

experimental protocols to permit accurate enumeration of the persister fraction (Figure 3.27).

Exposing bacteria to a lethal antibiotic dose followed by enumeration of survivors permits rapid characterization of the persister fraction. This approach was undertaken to assess the ability of antibiotics to modulate persister formation in uropathogens. Both *E. coli* CFT073 and *S. saprophyticus* 15305 showed significant ($P < 0.05$; $P < 0.01$ respectively) increases in percent survival following ciprofloxacin, ampicillin, and gentamicin pre-treatment and challenge (Figure 3.28). Analysis of strains from these surviving fractions did not reveal any significant changes in MIC compared to the original starting culture, indicating that spontaneous resistance which developed during sub-inhibitory antibiotic challenge did not contribute to survival (data not shown).

Resuscitation of persisters following therapy cessation presumably plays a critical role in antibiotic therapy failure. Therefore, the recalcitrance rate of persisters was evaluated in the presence or absence of antibiotic priming. This was accomplished by reconstituting presumed 'killed' cultures in fresh media and observing persister re-emergence via turbidity. There was a significant difference in persister resuscitation time for *E. coli* CFT073 compared to *S. saprophyticus* 15305, with the former emerging rapidly (exclusively within 24 hours) across treatment groups while the latter required ~24-72 hours depending on the antibiotic (Figure 3.29). The antibiotic priming influenced resuscitation rates in *S. saprophyticus* 15305 but not *E. coli* CFT073, with ciprofloxacin and gentamicin pre-treatments prolonging reactivation. In some cases, persister cells could spontaneously emerge up to 14 days following initial challenge in *S. saprophyticus* 15305 but not *E. coli* CFT073 cultures.

Figure 3.27. Comparison of growth kinetics for all uropathogens tested. Means are from at least two independent experiments. Significance was determined using unpaired t-test (all *E. coli* strains are non-significant for growth variation over a 24 hour period; $P > 0.05$).

Figure 3.27

OD600
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (hours)

TOP277
TOP263
TOP379
TOP344
TOP345
PUTS277
PUTS278
PUTS1127
PUTS1236
UTI89
UTI89 $LexA_{T355G}$
UTI89 ∆recA
CFT073
SS 15305

Figure 3.28. Persister induction following a 1/4MIC pre-treatment with either ciprofloxacin, ampicillin, or gentamicin for 3 hours. Percent surviving fractions represent the persister sub-population following 3 hour lethal treatment with either ciprofloxacin (A, D), ampicillin (B, E), or gentamicin (C, F) in *E. coli* CFT073 (A-C) or *S. saprophyticus* 15305 (D-F). Means from at least three independent experiments shown with significance. Significance determined using unpaired t-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 3.28

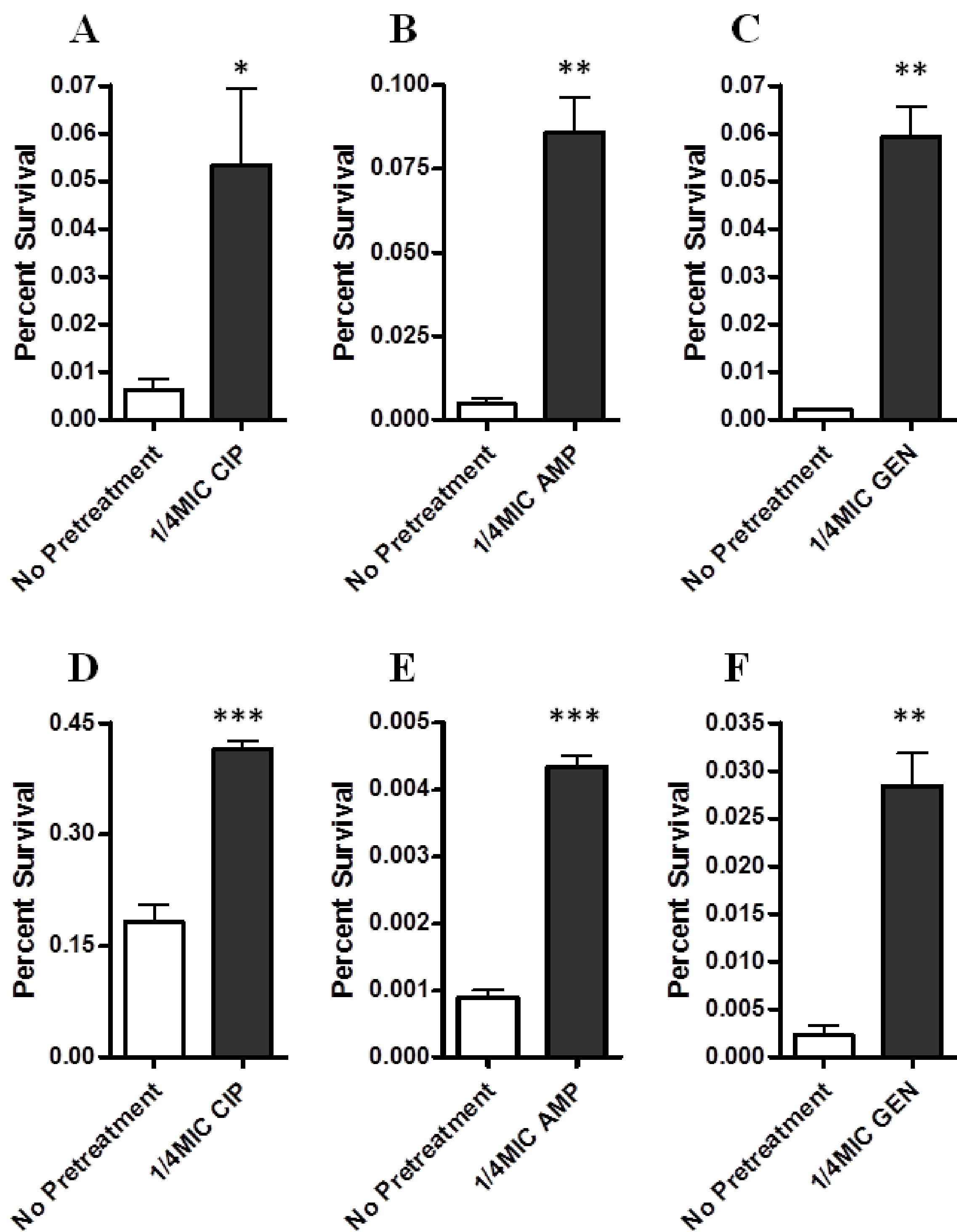
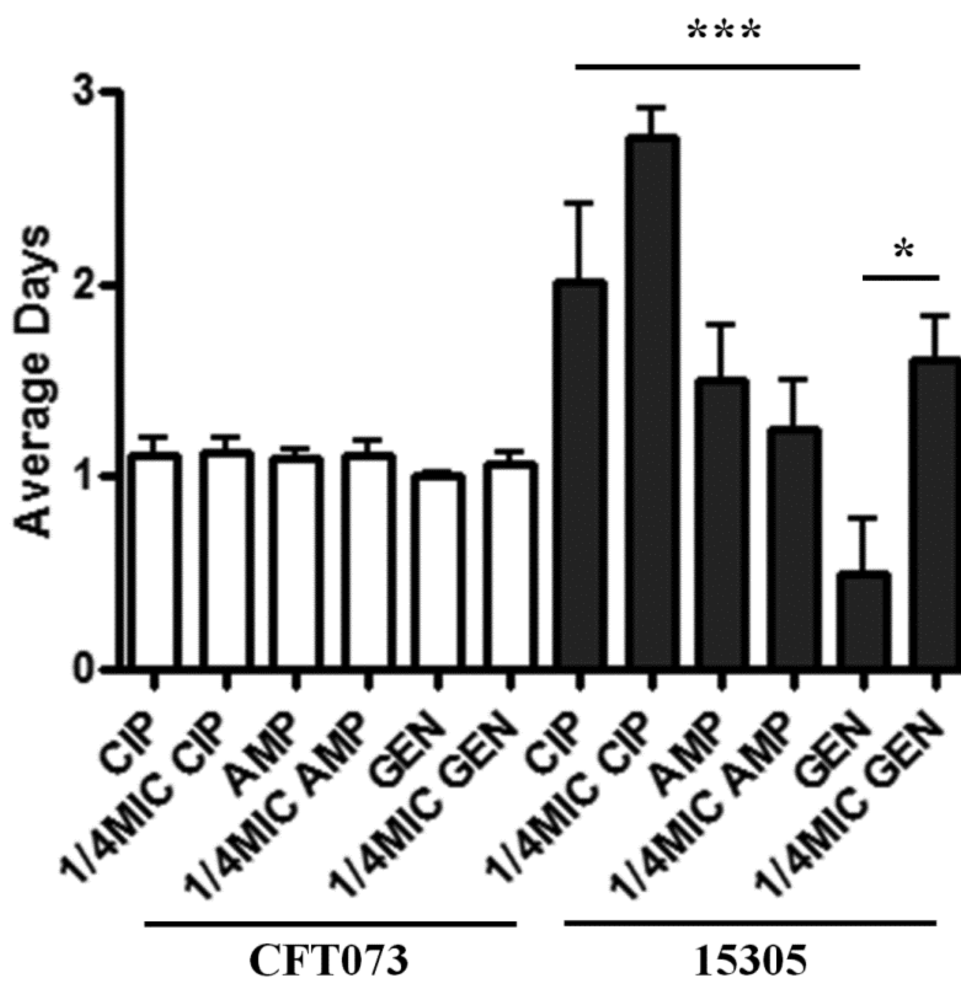


Figure 3.29. Average number of days till persister resuscitation following lethal antibiotic challenge. Pre-treated (1/4MIC) and non-treated cultures were considered for both *E. coli* CFT073 and *S. saprophyticus* 15305. Means from at least four independent experiments shown with significance. Significance was determined using one-way ANOVA and Bonferroni's multiple comparison test (*, $P < 0.05$; ***, $P < 0.001$).

Figure 3.29



3.3.2 Evaluating antibiotic susceptibility patterns in Type I and II persister cells

The efficacy of ciprofloxacin, ampicillin, and gentamicin was surveyed against stationary phase (predominantly Type I persister) cultures. *S. saprophyticus* 15305 demonstrated significantly greater antibiotic tolerance during stationary phase with percent survival increasing 443.2, 90,263.0 and 14,682.4-fold compared to exponential phase cultures for ciprofloxacin, ampicillin, and gentamicin respectively ($P < 0.001$) (Figure 3.30B). Similarly, *E. coli* CFT073 survival increased 676.2, 5,325.0, and 7,782.1-fold following lethal ciprofloxacin, ampicillin, and gentamicin challenge ($P < 0.001$) (Figure 3.30A). Heterogeneity in percent survival was observed across bacterial genera and antibiotic class. *S. saprophyticus* 15305 was significantly more susceptible to gentamicin (33.5% survival) than ciprofloxacin (80.9%) or ampicillin (80.7%) ($P < 0.001$). Conversely, *E. coli* CFT073 demonstrated greater survival against ampicillin (76.8% survival) and gentamicin (56.6%), while remaining significantly susceptible to ciprofloxacin (3.6%) ($P < 0.001$).

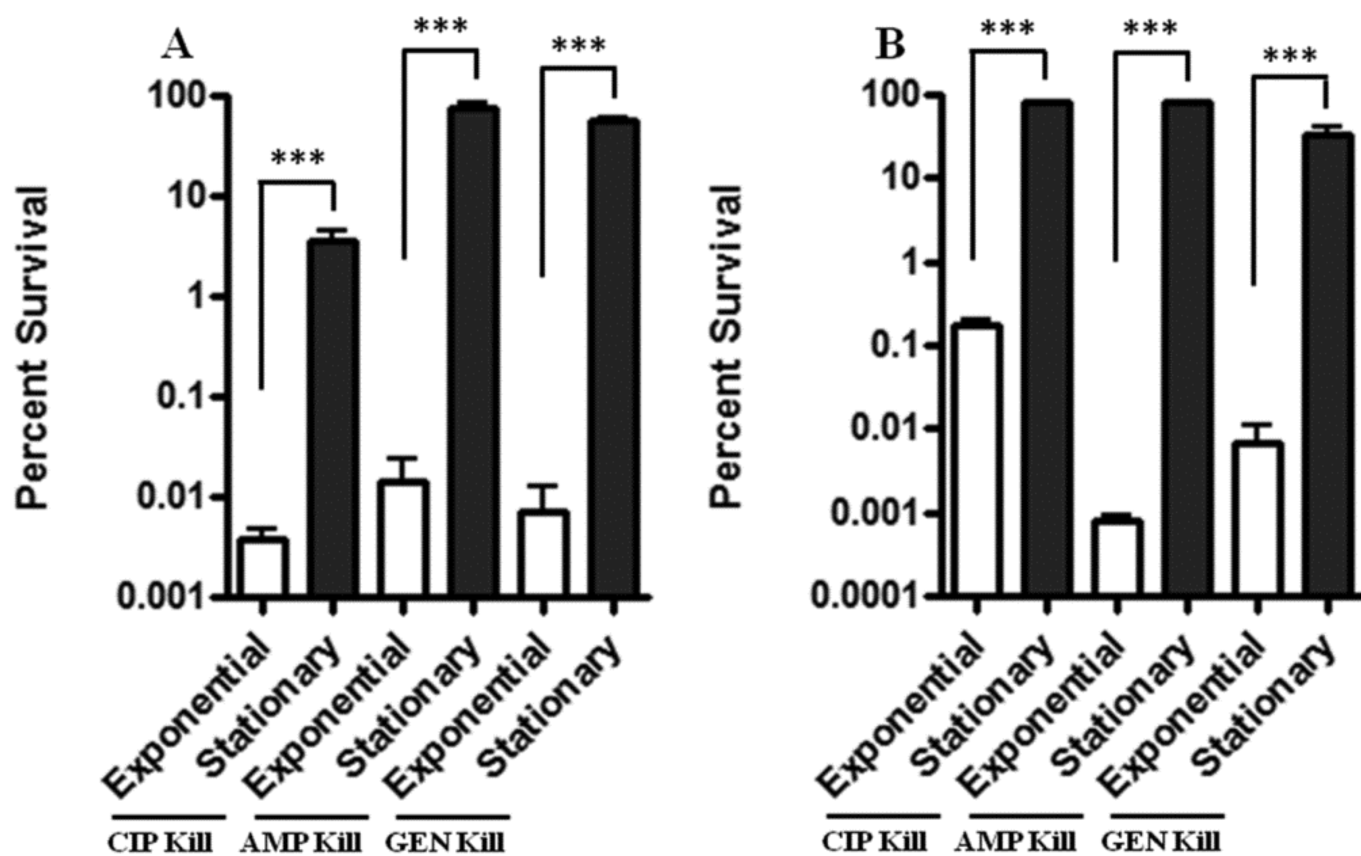
3.3.3 Characterizing the nature of antibiotic-induced persisters by challenging with lethal doses of the same and different agents

The large variations observed in persister fraction size between antibiotic treatment groups suggests heterogeneity in persister sensitivity to antibiotics. This finding discredits the null hypothesis that dormancy is both necessary and sufficient to convey MDT, such that persisters will respond to different antibiotics with the same levels of survival. It was therefore hypothesized that antibiotic-induced persisters would demonstrate similar heterogeneity, implying that dormancy is not a significant contributing factor of MDT.

The nature of the antibiotic-induced persister fraction was explored by challenging with the same agent that induced tolerance in addition to those with different cellular targets. Cross-tolerance was detected for some antibiotic classes but varied with organism genus. Ciprofloxacin and gentamicin pre-treatments most effectively enhanced survival of

Figure 3.30. Fraction sizes of Type I and Type II persisters of exponential and stationary phase cultures. Percent survival was determined following 3 hour challenge with lethal ciprofloxacin, ampicillin, or gentamicin for both *E. coli* CFT073 (A) and *S. saprophyticus* 15305 (B). Means from at least three independent experiments are shown with significance. Significance was determined using one-way ANOVA and Bonferroni's multiple comparison test (***, $P < 0.001$).

Figure 3.30



E. coli CFT073 (Figure 3.31A, C), while ampicillin pre-treatment was favourable in *S. saprophyticus* 15305 (Figure 3.31). Sub-inhibitory ciprofloxacin significantly enhanced survival 5.2-fold and 3.2-fold following lethal treatment with ampicillin and gentamicin in *E. coli* CFT073, while sub-inhibitory ampicillin significantly increased survival 4.1-fold and 34.9-fold following lethal ciprofloxacin and gentamicin challenge in *S. saprophyticus* 15305 ($P < 0.05$; $P < 0.01$). The variability of surviving fraction sizes between sub-inhibitory antibiotic pre-treatment groups further recapitulates the hypothesis that dormancy does not solely account for MDT. However, the relative contribution of uropathogen persister cells to UTI recurrence risk, and their capacity to form in the presence of sub-inhibitory antibiotic doses, are not well documented and warrant investigation.

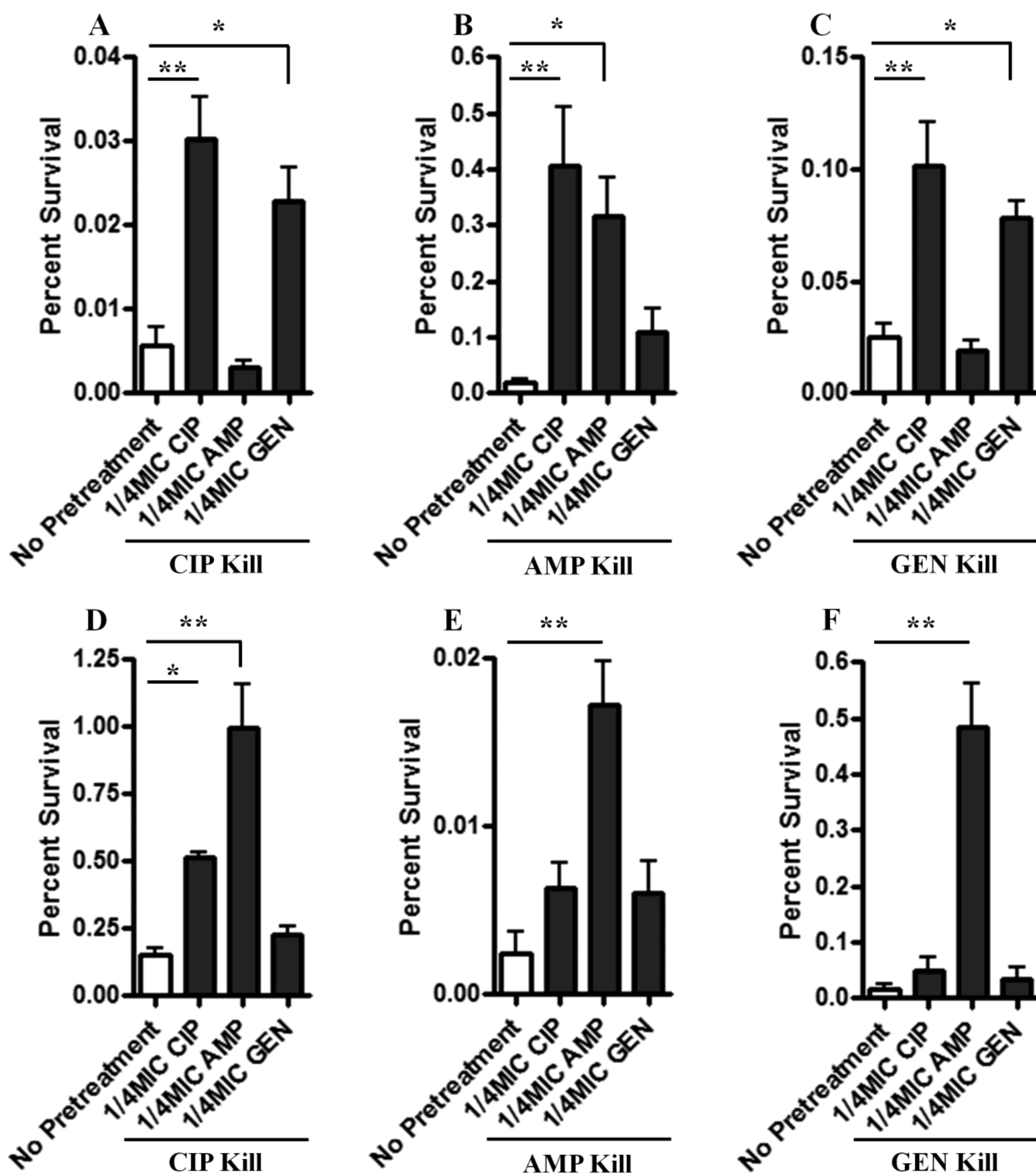
3.3.4 Characterizing the persister fraction of *E. coli* strains isolated from same-strain recurrent and acute infections

A direct correlation between persister fraction size and recurrence risk has never been explored. However, the MDT characteristics of persister cells potentiates their role in recurrent UTI. Although MDT is acquired through physiological changes, the persister fraction of an organism is genetically ordained, suggesting that these traits are subject to selective pressure during antibiotic therapy. This property was explored by assessing the persister forming capacity of *E. coli* strains isolated from patients suffering from either typical acute infection (AI) or same-strain recurrence (SSR).

As growth phase influences the persister fraction, we first confirmed that all UPEC strains demonstrated similar rates of growth (Figure 3.27). Thus, any change in the persister fraction is due solely to variations in the organism's capacity to form persisters. Stationary phase cultures were considered for Type I persister frequency. Standard antibiotic susceptibility testing revealed all strains to be similarly susceptible to the antibiotics tested, with the exception of SSR organisms TOP379 and TOP344 which demonstrated intermediate susceptibility to ampicillin (Table 3.1). Similar to *E. coli* CFT073, all isolates within the Type I stationary fraction tolerated ampicillin and gentamicin while succumbing to ciprofloxacin treatment (Figure 3.32). Unexpectedly,

Figure 3.31. Cross-tolerance susceptibility patterns of Type II persisters induced with various antibiotic pre-treatments. Percent surviving fractions represent the persister sub-population following 3 hour lethal treatment with either ciprofloxacin (A, D), ampicillin (B, E), or gentamicin (C, F) for both *E. coli* CFT073 (A-C) and *S. saprophyticus* 15305 (D-F). Means from at least three independent experiments are shown with significance. Significance was determined using one-way ANOVA and Bonferroni's multiple comparison test (*, $P < 0.05$; **, $P < 0.01$).

Figure 3.31



some SSR organisms revealed slight growth during lethal treatment. Although antibiotics were already provided at therapeutic levels, we observed the same result upon challenging with 10x the previous lethal dose (data not shown). Viability of all AI strains declined as expected. When grouped, AI strains had significantly lower survival for ampicillin (69.15%) and gentamicin (77.93%) treatment compared to SSR organisms (118.0% and 151.2% respectively), which had a higher persister fraction ($P < 0.01$). Surviving organisms did not display any changes in MIC following the experiments, suggesting spontaneous acquisition of resistance elements did not occur (data not shown).

Cross-tolerance assays were also conducted to determine sub-inhibitory antibiotic effects on Type II persister formation during exponential phase growth and to permit assessment of their susceptibility. Ciprofloxacin and gentamicin pre-treatments induced persister states at the highest frequency in SSR organisms ($P < 0.05$; $P < 0.01$; $P < 0.001$) (Figure 3.33A), while most AI strains failed to respond to antibiotics (Figure 3.33B). However, gentamicin pre-treatment significantly ($P < 0.01$) increased the persister fraction in *E. coli* AI strain PUTS1236. In almost all cases, pre-treatment with ampicillin significantly ($P < 0.05$) improved the efficacy of gentamicin in both SSR and AI organisms. Some antibiotic/SSR-organism combinations again demonstrated slight growth in lethal antibiotic concentrations. This effect was amplified in strains TOP379 and TOP344 which are intermediately susceptible to ampicillin, with growth observed following lethal treatment with ampicillin and gentamicin irrespective of pre-treatment compared to non-treated controls. Taken together, these results indicate that organisms with large persister fractions either initiate recurrent UTI, or that persister traits are selected for during repeated bouts of antibiotic therapy.

3.3.5 SOS-deficient *E. coli* persister cells are not tolerant to ciprofloxacin

The involvement of SOS response systems and virulence enhancement has been a recurring theme of this work. SOS responses have been intrinsically linked with the formation of persister cells following fluoroquinolone therapy, which is a potent DNA damaging agent and therefore stimulator of this system. These observations permit

Figure 3.32. Percent survival of same strain recurrent (SSR) and acute infection (AI) *E. coli* isolates challenged with lethal ciprofloxacin (A), ampicillin (B), or gentamicin (C) during stationary phase growth. Dashed lines indicate average SSR or AI grouped survival to the respective antibiotics. Means from at least four independent experiments are shown with significance. Significance between SSR and AI groups was determined by comparing average survival using an unpaired t-test (ns = not significant; **, $P < 0.01$).

Figure 3.32

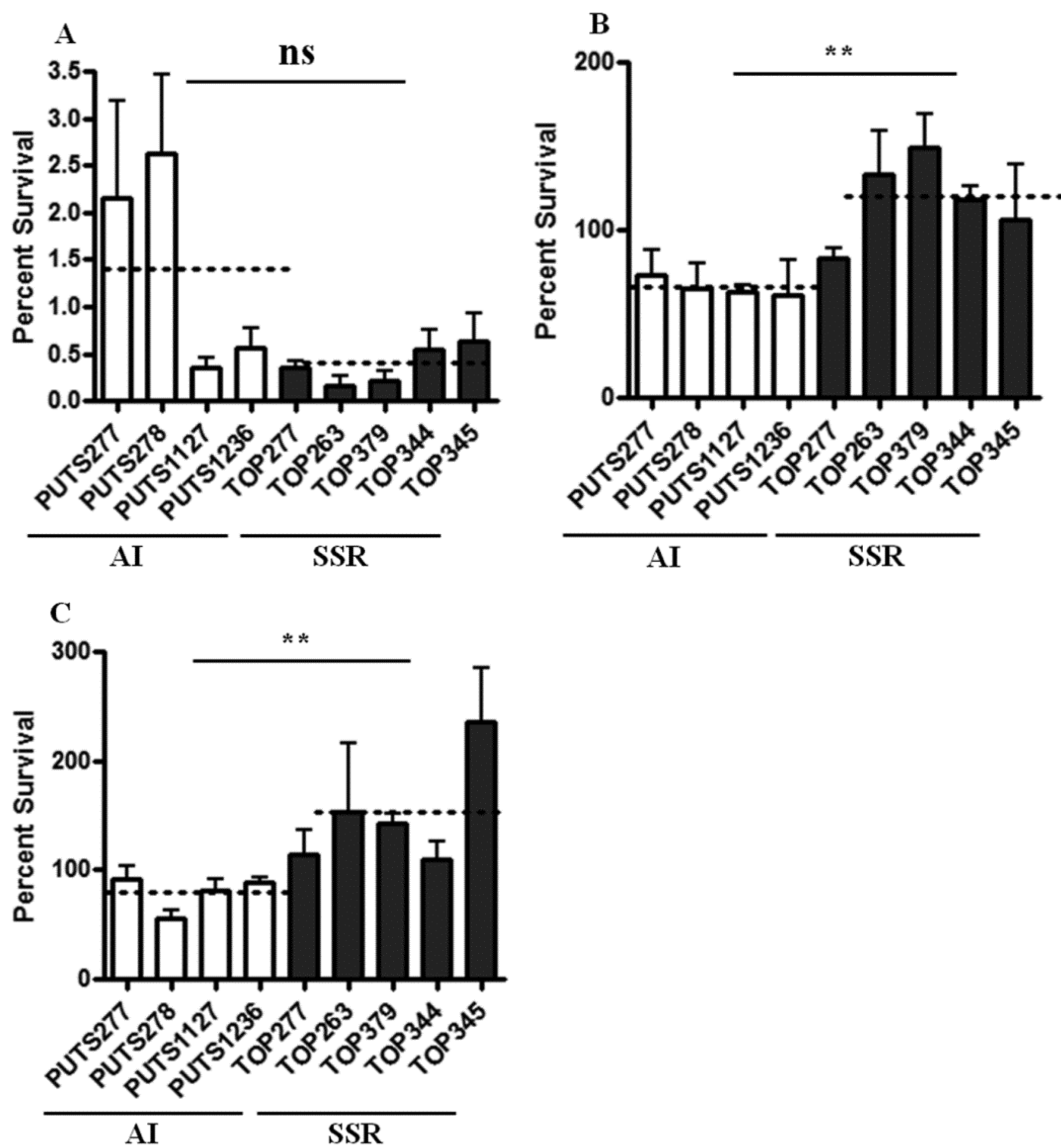


Figure 3.33. Cross-tolerance susceptibility patterns of SSR (A) and AI (B) *E. coli* persisters induced with various antibiotic pre-treatments. Percent survival was determined after challenge with a lethal dose of either ciprofloxacin, ampicillin, or gentamicin. Means from at least four independent experiments are shown with significance. Significance was determined using one-way ANOVA and Bonferroni's multiple comparison test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 3.33

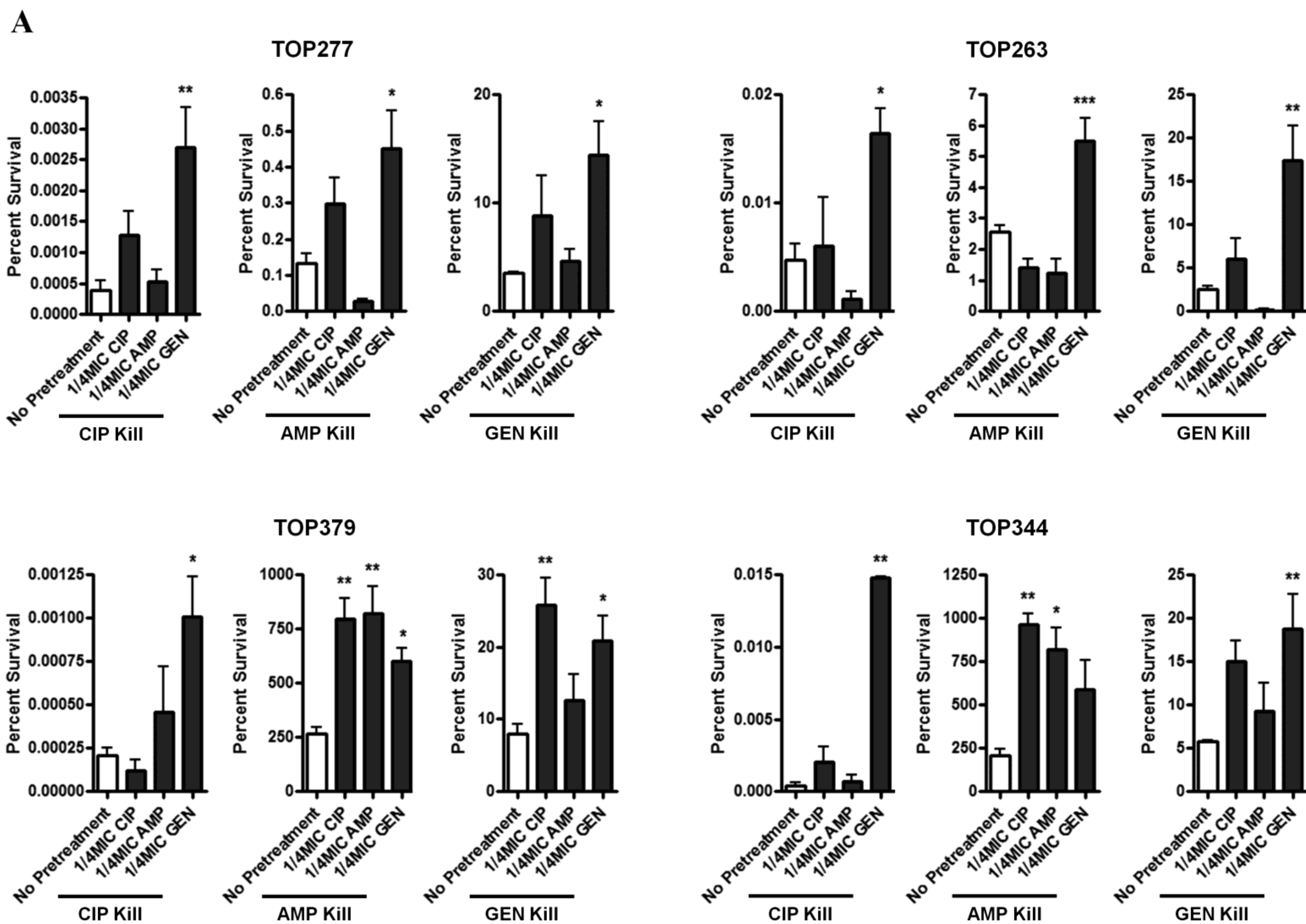
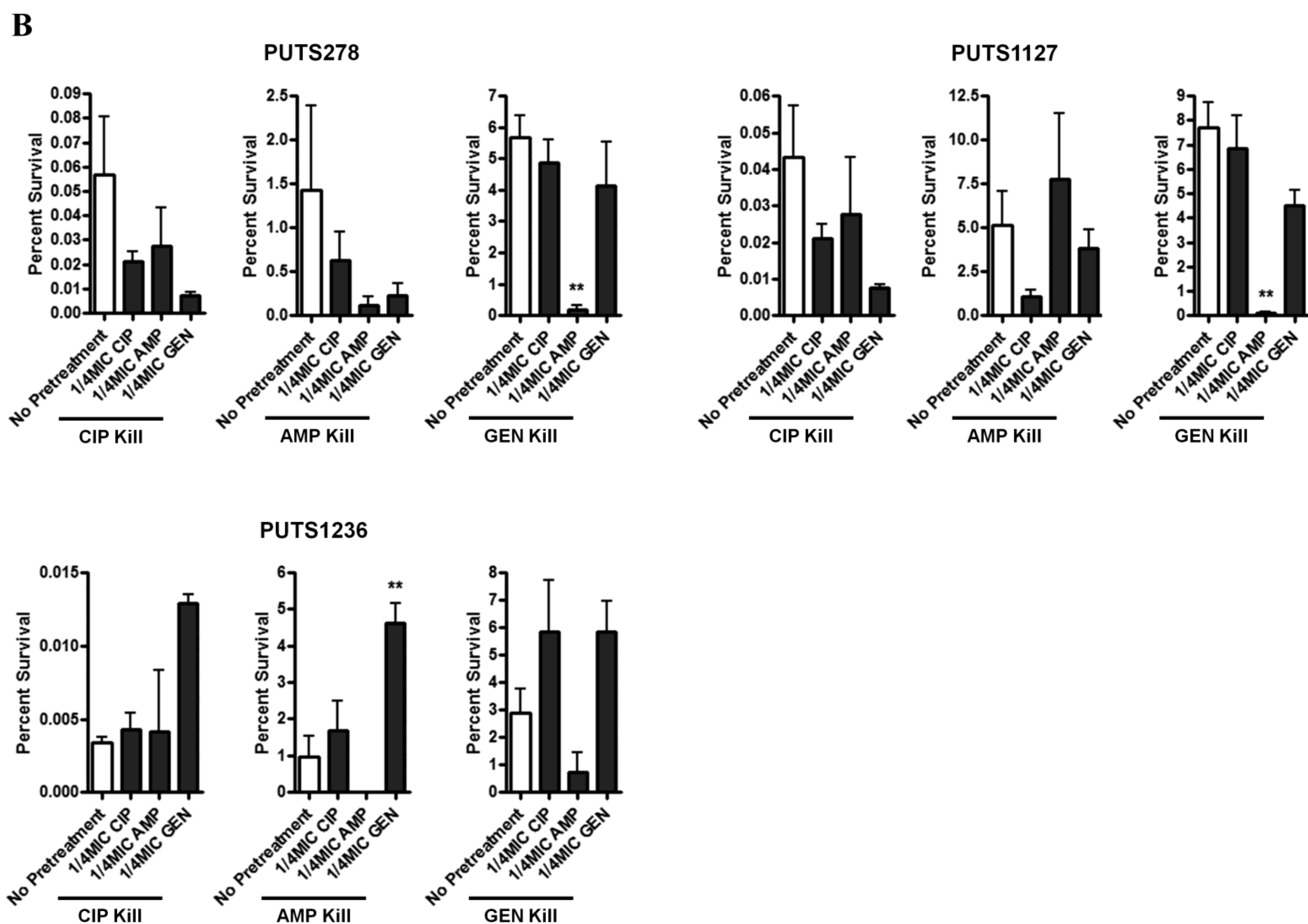


Figure 3.33 (continued)



further evaluation of the role dormancy plays in MDT. It was hypothesized that if complete metabolic dormancy is both necessary and sufficient for MDT then established persisters should be refractory to antibiotics regardless of the functionality of their SOS system. Investigation of the SOS system's contribution to persister onset with other antibiotics was also warranted.

E. coli UTI89 strains deficient in RecA production (Δ*recA* - PAS0209) and LexA autoproteolysis ($lexA_{T355G}$ - PAS0211) were considered in order to compare the effects of insufficient double-strand break repair and SOS-induction on persister cell maintenance and onset. *E. coli* UTI89 strains PAS0209 and PAS0211 were significantly ($P < 0.001$) sensitized to ciprofloxacin challenge, thus 1/4MIC pre-treatment values were adjusted accordingly (Table 3.1). Ciprofloxacin and gentamicin priming significantly induced persister cell production in wild-type *E. coli* UTI89 as anticipated ($P < 0.01$; $P < 0.001$) (Figure 3.34A-C). This effect was completely abrogated in both SOS-deficient strains regardless of pre-treatment and subsequent lethal dose applied (Figure 3.34A-C). Although less proficient in antibiotic-dependent persister induction, SOS-deficient *E. coli* strains PAS0209 and PAS0211 did produce Type II persister levels that were comparable to those of wild-type *E. coli* UTI89.

The contribution of active double-strand break repair and SOS response to antibiotic tolerance was investigated using the Type I, stationary phase persister fraction. The surviving fractions of the SOS-deficient *E. coli* UTI89 strain PAS0211 was significantly reduced 29.7-fold following ciprofloxacin challenge ($P < 0.001$) (Figure 3.35). In addition, *E. coli* UTI89 strain PAS0209 which is devoid of RecA activity, was completely eradicated (to the limit of detection) following ciprofloxacin challenge ($P < 0.001$). However, persister levels were unchanged following incubation with ampicillin and gentamicin compared to wild-type *E. coli* UTI89 indicating PAS0209 and PAS0211 strains were not deficient in persister cell development. These finding suggest that topoisomerases II and IV are not only active within persisters, but corruptible with ciprofloxacin treatment. Furthermore, the observed eradication of *recA* knock-out strains indicates that active double-strand DNA break repair is required for tolerance. It is reasonable to conclude with this data that persister cells do not constitute completely metabolically inactive variants.

Figure 3.34. Cross-tolerance assays comparing persister induction capacity of wild-type or SOS-deficient *E. coli* UTI89 strains following subinhibitory antibiotic therapy. Percent survival was determined after challenge with a lethal dose of either ciprofloxacin (A), ampicillin (B), or gentamicin (C). Means from at least five independent experiments are shown with significance. Significance was determined using one-way ANOVA and Bonferroni's multiple comparison test (ns = not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 3.34

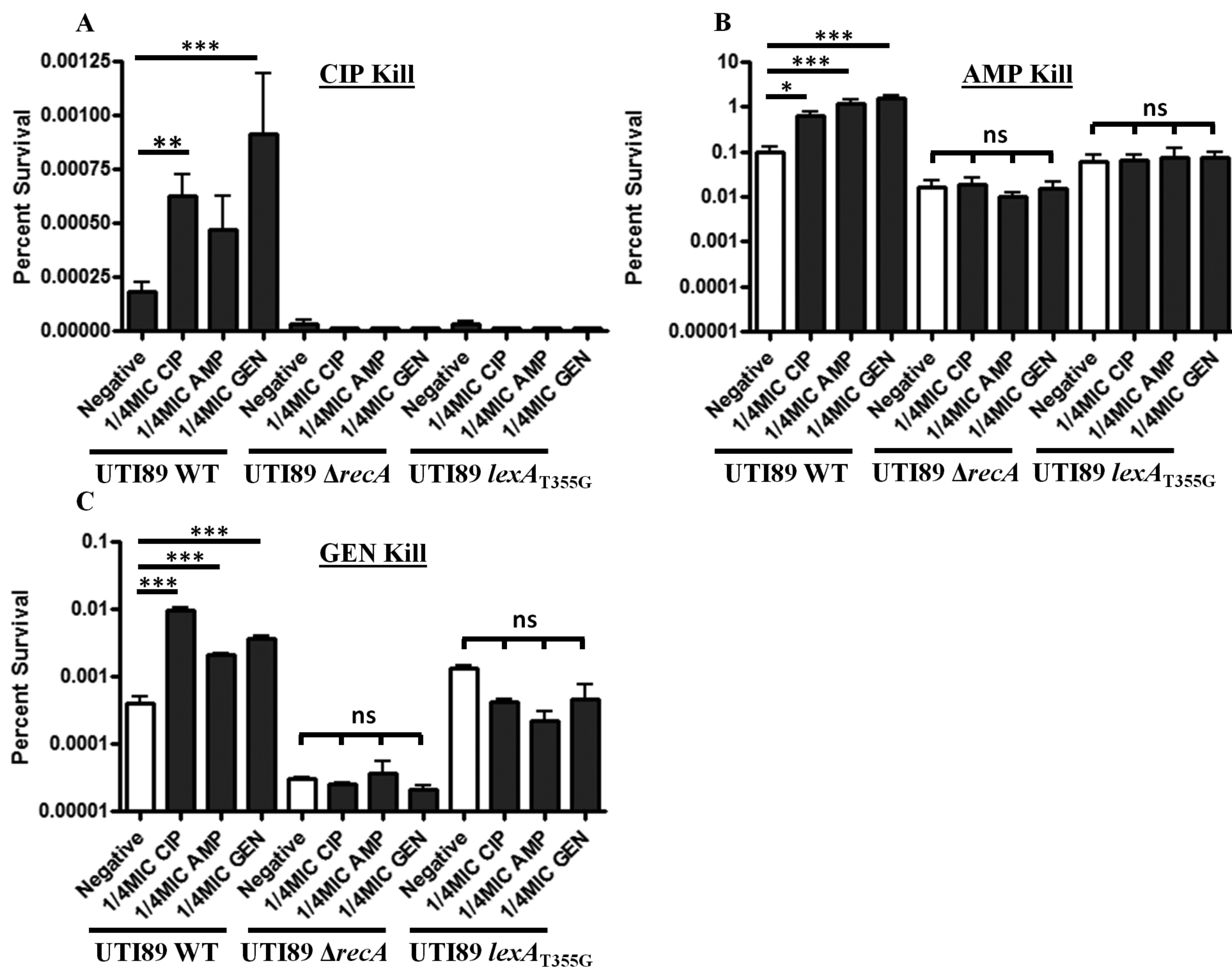
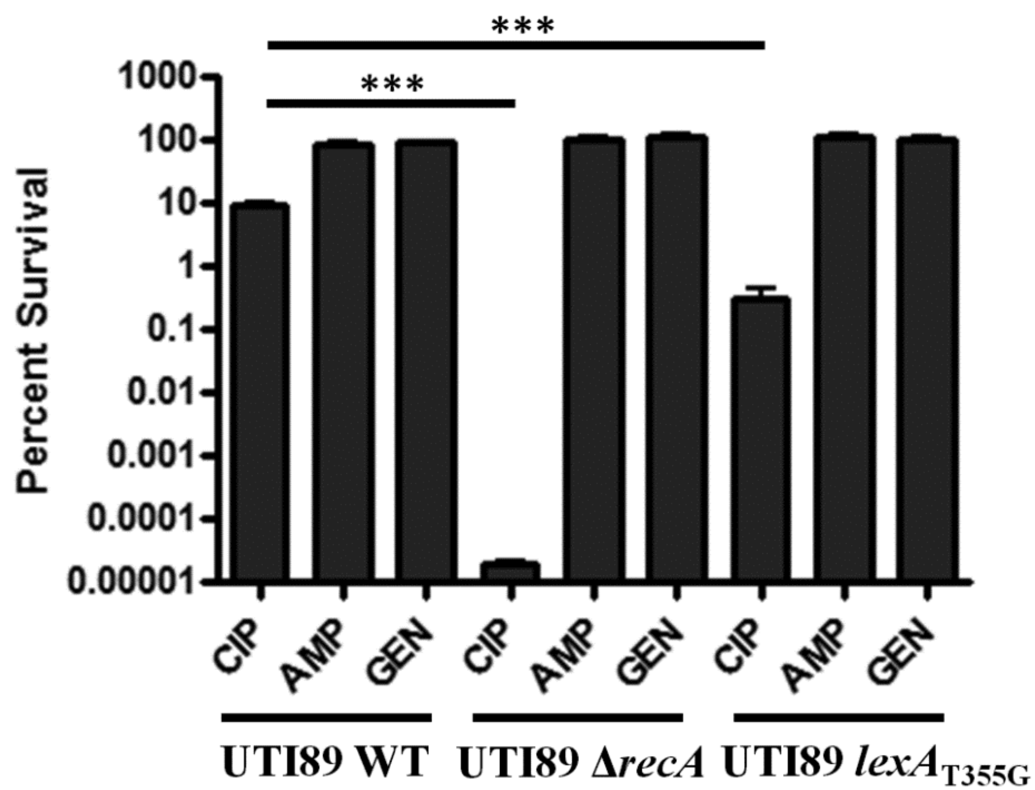


Figure 3.35. Sensitivity of Type I *E. coli* persisters deficient in SOS to various antibiotics. Fraction size was determined following 3 hour challenge of stationary phase cultures with lethal ciprofloxacin, ampicillin, or gentamicin and enumerating the surviving population. Means from at least four independent experiments are shown with significance. Significance was determined using one-way ANOVA and Bonferroni's multiple comparison test (***, $P < 0.001$).

Figure 3.35

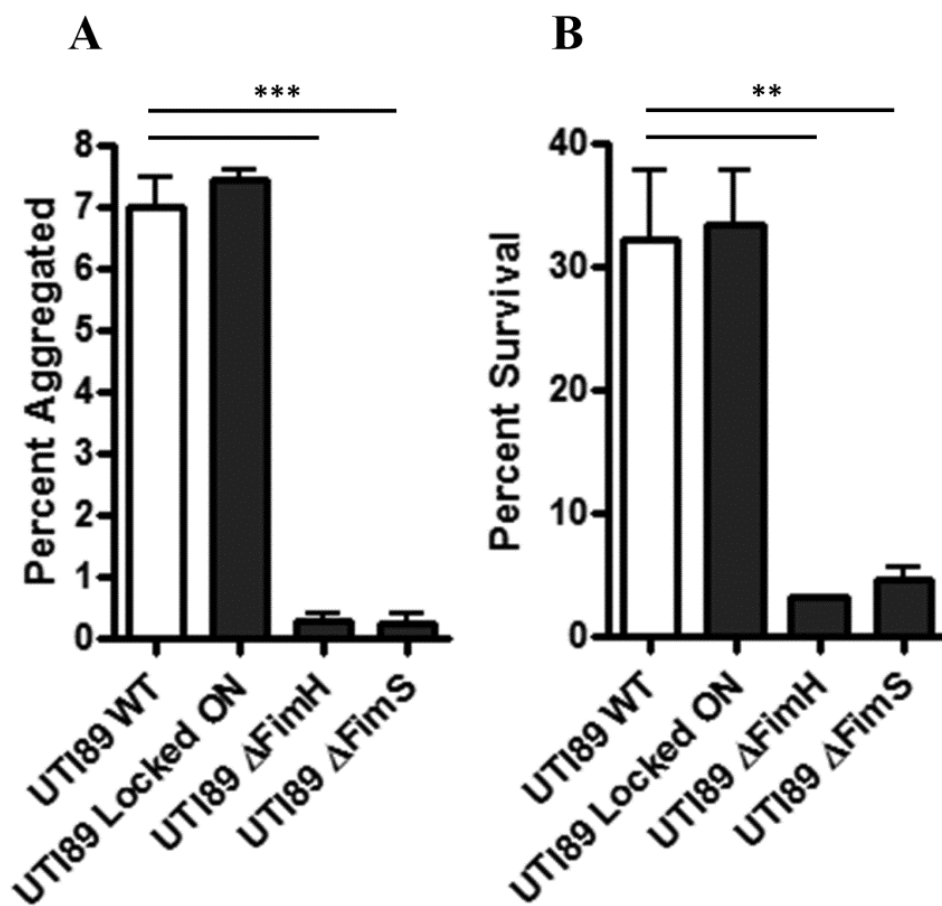


3.3.6 Assessing the contribution of high-density growth to persister cell onset

As sub-inhibitory antibiotics promote aggregation (Figure 3.8) and persister formation (Figures 3.28, 3.31, 3.33), it was hypothesized that there may be a link between these two processes and MDT onset. This was tested using stationary phase cultures of *E. coli* UTI89 deficient in aggregation which is conferred through atypical type 1 fimbriae formation. Aggregation was assessed using a sediment assay which measures the rate of settling over time as a function of aggregate size. Type 1 fimbriae deficient strains Δ*fimH* (fimbriae expressed without adhesin) and Δ*fimS* (fimbriae not expressed - bald phenotype) were utilized. As expected, wild-type and hyper-fimbriated (Locked ON) *E. coli* UTI89 strains demonstrated significant aggregation while aggregation-deficient strains did not ($P < 0.01$) (Figure 3.36A). Stationary phase killing assays were then carried out and revealed that aggregation-deficient strains were also significantly deficient in persister formation, demonstrating ~10-fold fewer survivors than wild-type *E. coli* UTI89 ($P < 0.01$) (Figure 3.36B). Although direct cause-and-effect relationships cannot be confirmed from these results, the data presented throughout this work is compelling evidence that high-density growth may be a trigger for the induction of persister states.

Figure 3.36. Percent aggregation of stationary phase, wild-type, Locked ON, Δ*fimH*, and Δ*fimS E. coli* UTI89 strains (A), and the subsequent persister fractions of each strain following exposure to lethal ciprofloxacin for 3 hours. Means from at least three independent experiments are shown with significance. Significance was determined using one-way ANOVA and Bonferroni's multiple comparison test (**, $P < 0.01$; ***, $P < 0.001$).

Figure 3.36



CHAPTER FOUR

DISCUSSION

4 DISCUSSION

4.1 THE IMPORTANCE OF UNDERSTANDING THE INFLUENCE OF ANTIBIOTICS ON THE HOST AND PATHOGEN

Antibiotics have been used successfully in treating bacterial infections for over 60 years. In this time, humanity has seen their canonization from modest beginnings as soil-derived signalling agents to clinically available cures, saving countless lives. However, the 'post antibiotic era' is already quickly becoming a reality, in part driven by indiscriminate, inappropriate and promiscuous use. Well-respected researchers have drawn attention to some of these issues in recent years (Levy and Marshall, 2004), calling for the global banning of antibiotic use for animal weight-gain, over-the-counter sales in developing countries, and demanding stricter prescribing policies for physicians and dentists (Angulo *et al.*, 2005; Kay, 2012; Ali *et al.*, 2006). Models suggest that even a 1% reduction in the usefulness of existing antibiotics, driven by inappropriate use, corresponds with costs of $600 billion to $3 trillion in lost human health (Hollis and Ahmed, 2013). Aspects of human nature are probably reflected in this antibiotic story. When something is so effective at a particular job, in this case killing pathogenic bacteria, humans have a tendency to rapidly, exhaustively and broadly apply the discovery often without completely understanding the consequences of such promiscuous use. Thus, antibiotics are still administered for the common cold, despite it being caused by a virus (Pechère, 2001); they are prescribed prophylactically for patients undergoing many surgical and dental procedures when risk of infection is low and antibiotic efficacy weak (Enzler *et al.*, 2011); they are utilized in children repeatedly and often for long periods of time, in part because diagnostic tools are limited or poor, or physicians are guided by ‘instinct’ rather than evidence-based findings (Conway *et al.*, 2007).

It is now coming to light that issues associated with antibiotics extend far beyond those related to drug resistance. Only within the last decade have scientists began to understand the natural role of antibiotics in the environment (Linares *et al.*, 2006; Romero *et al.*, 2011; Yim *et al.*, 2007). Newly discovered inter-species signalling properties may

potentiate the augmentation of bacterial virulence when applied therapeutically. To complicate matters, some antibiotic classes are also known to directly influence the host, necessitating even further characterization to optimize treatment strategies (Dalhoff and Shalit, 2003; Čulić *et al.*, 2001). These findings raise several important questions regarding contemporary antibiotic use in the clinic. Firstly, are antibiotics being applied unnecessarily to certain patient cohorts? If so, does their application negatively affect treatment outcome? Secondly, can the signalling properties of antibiotics on the host and pathogen be capitalized on to enhance therapeutic efficacy? Thirdly, do different antibiotic classes confer different phenotypic properties in different pathogens? The studies conducted here aim to answer these questions in the context of antibiotic therapy for the treatment of recurrent UTI. This disease milieu presents an ideal platform for the study of antibiotic management strategies, as there is growing debate over the necessity of its application and associated methodologies (Garin *et al.*, 2006; Nickavar and Sotoudeh, 2011). In this thesis, the effects associated with inadequate antibiotic therapy are demonstrated, and reveal that multiple aspects of bacterial virulence are modulated affecting disease outcome. Ultimately, understanding the influence of antibiotics on the host, pathogen, and infection outcomes will provide physicians with new strategies for therapeutic optimization, and knowledge that helps them avoid the use of antibiotics when they are not effective or even detrimental to the patient.

4.2 THE INFLUENCE OF SUB-INHIBITORY ANTIBIOTICS ON UROPATHOGEN VIRULENCE

Studies characterizing the wider effects of sub-inhibitory antibiotics on soil organisms and human pathogens are still in their infancy. Thus far, techniques have focused primarily on the use of transcriptomic approaches, both for their high-throughput potential and ready application to a wide variety of organisms (Linares *et al.*, 2006; Fajardo and Martínez, 2008). Although these studies have provided the first links between off-target antibiotics effects on bacteria and their potential contribution to disease, they are not ideal for the study of pathogenesis as changes in gene transcription do not often reflect changes in translation, protein folding or extracellular localization

(Straub, 2011). Therefore, one of the aims of this thesis was to characterize changes in virulence expression of uropathogens at the phenotypic level.

4.2.1 Effect of sub-inhibitory antibiotics on uropathogen adhesin expression and adherence to surfaces

The capacity for antibiotics to affect bacterial adherence to abiotic and biotic surfaces were characterized. This was initially done by assessing the ability of *S. saprophyticus* 15305 to adhere to the surface of glass microscope slides in the presence and absence of antibiotics. Human pooled urine (HPU) was used as a growth substrate over routine culture media for its capacity to form conditioning films on contact with foreign surfaces (Santin *et al.*, 1999; Canales *et al.*, 2009). Although more relevant to the urinary tract, the use of HPU posed several problems, the foremost of which was poor inter-experimental reproducibility due to batch variation. This was despite steps being taken to avoid such issues, which included strict collection schedules and extraction from the same donors. However, the metabolic and protein profiles of urine are known to change over the course of a day, month, and year (Connor *et al.*, 2004). In addition, diet, medication, and hormone levels heavily influence urinary composition and could not be controlled in this study. For these reasons, the use of this medium was thereafter discontinued.

Experiments investigating the adherence of *S. saprophyticus* 15305 to glass microscope slides demonstrated that sub-inhibitory antibiotic exposure influenced this process. Ciprofloxacin, ampicillin, and gentamicin were all capable of increasing bacterial adherence to similar degrees despite targeting different cellular machinery. Although not conclusive, this finding suggests that observed induction of adherence is mediated through general stress response systems. Activation of the Sigma B transcription factor by fluoroquinolones has been demonstrated to increase adhesion in quinolone-resistant strains of *S. aureus*, but other antibiotics and response systems have not been explored in non-resistant strains (Li *et al.*, 2005[a]).

As glass exhibits hydrophilic surface chemistry not reflective of materials or tissues routinely found in the urinary tract, hydrophobic medical devices utilized in this

environment were subsequently investigated. This is an important consideration, as hydrophilic surfaces are poorly colonized by most bacteria due to the repulsive interaction of negatively charged elements (Boks *et al*., 2008). In addition to their experimentally desirable surface properties, stents and catheters are also widely used in urological applications and are usually readily colonized (Habash and Reid, 1999; Siddiq and Darouiche, 2012). Together these materials represent the greatest cause for nosocomial infection, and are associated with increased morbidity, hospital cost, and length of stay, further warranting their use in this model (Saint, 2000; Platt *et al.*, 1982; Givens and Wenzel, 1980; Tambyah *et al.*, 2002). Antibiotic priming of *S. saprophyticus* 15305 resulted in greater adherence to both stent and catheter material. The change in adherence was more substantial than found with glass microscope slides, supporting the adhesion promoting properties of these devices. The hydrophobic surface chemistry of urological devices is often masked using hydrophilic, hydro-gel coatings for this reason (Beiko *et al.*, 2004). Catheter colonization was higher than stent colonization, likely due to the different polymers used between these materials. The proprietary polymer utilized in the InLay stent prohibits inquiries into its anti-infective properties. However, the latex polymer surfaces used in Foley catheters are more irregular, and readily colonized compared to silicone material which support the findings presented (Nickel and Costerton, 1992; Stickler *et al.*, 2003).

The capacity for different antibiotic classes to promote urological device colonization is concerning. Single dose antibiotics are usually applied prophylactically during device placement as per American Urological Association guidelines, a practice which may not be warranted (2008), and might actually promote adhesion. In addition, the modification of urological materials through coating, matrix loading, and immersion in antimicrobial agents has become a widespread practice (Siddiq and Darouiche, 2012). Many coatings have become available, and incorporate bactericidal silver ions and nanoparticles, enzyme inhibitors, antibiotics and sometimes even bacteriophages (Saint *et al*., 1998; Furno *et al.*, 2004; Morris and Stickler, 1998; Burton *et al.*, 2006; Carson *et al*., 2010; Mendez-Probst *et al*., 2012). The aim of these enhancements are to discourage pathogen growth once they have been driven into the urinary environment during device placement. However, the efficacy of modified devices has shown no benefit over

untreated instruments, questioning the merit of their application (Srinivasan *et al.*, 2006; Cadieux *et al*., 2009). This is especially true considering the capacity of antibiotics to improve bacterial device colonization, a process which could occur during long-term device placement once bio-active agents have been leached to sub-inhibitory levels.

Antibiotic-induced changes in *S. saprophyticus* 15305 adherence frequency to abiotic surfaces warranted further investigation into downstream processes such as modulation of biofilm formation. Biofilm-associated organisms are common during UTI, and play an important role in the contribution to recurrences, especially when urinary devices are present (Choong and Whitfield, 2000; Hatt and Rather, 2008; Reid *et al*., 1992). Organisms growing in the sessile environment of the biofilm matrix contribute to the aetiology, pathogenesis and continued persistence of bacteria in the urinary tract through antibiotic tolerance mechanisms and subversion of host defences including shear stress mediated detachment during voiding and anti-phagocytic properties (Nickel *et al.*, 1994; Donlan, 2002). In addition, biofilms continuously seed planktonic organisms into the urinary lumen contributing to disease propagation and spread to new environments (Costerton *et al.*, 1999).

The capacity for aminoglycosides to promote biofilm formation has been described, and is dependent on the Arr signalling pathway found in some *Enterobacteriaciae* (Hoffman *et al.*, 2005). Similar processes were explored in *S. saprophyticus* 15305 and *E. coli* UTI89 uropathogens in this thesis. All antibiotics tested induced biofilm formation for both uropathogens. *E. coli* UTI89 demonstrated greater biofilm accumulation compared to *S. saprophyticus* 15305, which may have been accounted for in part by aminoglycoside induction of the Arr signalling pathway. However, a search for this or other homologous genes in either organism using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) did not yield any results. The absence of this signalling system in *E. coli* UTI89 might be due to it being a human pathogen, and loss of this environmental response element through genome reduction via negative selection. Such processes have been demonstrated to increase fitness in site-specific, steady-state niches such as the human host, over those that are dynamically changing such as the soil environment (Koskiniemi *et al.*, 2012). Moreover, Arr was characterized in the soil bacterium and opportunistic pathogen *P. aeruginosa* PA01, supporting this system's

importance in environmental but not host niches (Hoffman *et al.*, 2005; Stover *et al.*, 2000). Antibiotic-dependent biofilm formation in these uropathogens could reasonably be driven through an alternative pathway, potentially governed by the activity of general stress response elements. Indeed, non-targeted stressors including nutrient deprivation, ethanol, and extremes in pH and salinity have all been implicated in driving biofilm formation (Fux *et al.*, 2005, Gotoh *et al*., 2010; Davey and O'toole, 2000; Knobloch *et al*., 2002; Lapaglia and Hartzell, 1997). An organism's commitment to sessile growth states in response to stress makes sense in the light of the reduced metabolic requirements and added matrix-associated protection imparted by biofilms (Kim *et al.*, 2009). However, the activity of specific antibiotic signalling systems cannot be ruled out at this time, especially from environmentally derived ampicillin and gentamicin. Although fluoroquinolones are fully synthetic compounds, they demonstrate quorum-sensing-like properties, which may influence biofilm development (Diggle *et al.*, 2003; Heeb *et al*., 2010).

The contribution of a general stress response system in antibiotic-dependent biofilm formation was tested using SOS-deficient strains derived from *E. coli* UTI89. Although there are several stress response systems in *E. coli*, SOS was considered because of its activation when exposed to several types of antibiotics (Miller *et al*., 2004; Mesak *et al.*, 2008). In addition, SOS has been implicated previously in the modulation of other virulence determinants (Kelley, 2006; Bisognano *et al*., 2000). Even without the application of antibiotics, both the Δ*recA* and $lexA_{T355G}$ strains tested had reduced biofilm forming capacity compared to wild-type *E. coli* UTI89, suggesting a role for SOS in normal biofilm formation, confirming previous studies (Gotoh *et al.*, 2010). These results were recapitulated with antibiotic treatment, whereby antibiotic-dependent biofilm formation was severely depressed or non-existent in both SOS-deficient strains. Gentamicin was capable of inducing biofilm formation in the $lexA_{T355G}$ but not Δ*recA* strain, suggesting downstream signalling via activated RecA, or its interaction with other cellular components might be involved. In addition, all strains were influenced by ampicillin regardless of the activity of their SOS response systems. These observations recapitulate findings which demonstrated low-dose β-lactam antibiotics enhance eDNA release in methicillin resistant and sensitive strains of *S. aureus*, resulting in the

production of more robust biofilms (Kaplan *et al.*, 2012). It was thought that autolysin activity was important in this process; however, such observations are absent in *E. coli* so their contribution to biofilm formation in this organism is speculative. Alternatively, ampicillin's activity at the cell surface might activate the Cpx two-component signal transduction pathway, which responds to disturbances in the cell envelope, in turn activating regulation of adhesion genes (Otto *et al*., 2002).

In addition to enhancing biofilm formation, antibiotics were able to induce rapid planktonic aggregation of both *E. coli* UTI89 and *S. saprophyticus* 15305. As with adherent biofilms, all antibiotic classes demonstrated similar capacity to induce aggregation. This phenotype may represent an alternative to biofilm formation when surfaces are not readily available. In this manner, uropathogen aggregates can benefit from the immuno-protective and antibiotic tolerance effects of sessile growth without committing to host cell adherence (Haaber *et al.*, 2012; Rakita *et al*., 1999). The mechanism of aggregation is likely conveyed through upregulation of polysaccharide intercellular adhesin (PIA) in *S. saprophyticus* 15305, which has been suggested through the use of a congo red agar plate assay (data not shown), and is an important mediator of biofilm formation in this species (Mack *et al*., 1994). Expression of this adhesin has been shown to be induced following antibiotic therapy and exposure to other stressors (Haaber *et al.*, 2012; Rachid *et al.*, 2000). Conversely, increased expression of type 1 fimbriae and other fimbrial-like surface components are likely constituents of aggregation in *E. coli* UTI89, and have been previously associated with adhesive, biofilm, and aggregative properties (Rodrigues and Elimelech, 2009; Schembri *et al.*, 2001). A semi-quantitative approach using TEM for enumerating fimbrial expression on *E. coli* UTI89 surface was attempted but did not yield any meaningful findings (data not shown).

Since uropathogen adherence to abiotic surfaces and biofilm formation were significantly upregulated in response to diverse classes of antibiotics, their capacity to induce adhesion to urothelial surfaces was also investigated. This was accomplished using the transitional epithelial carcinoma cell lines 5637 and A498, which were originally isolated from human bladders and kidneys, respectively. The use of these tissues was justified for their characteristics, extraction site, and previous *in vitro* UTI studies (Svensson *et al*., 2001; Mulvey *et al*, 2001). Antibiotic incubation with *S. saprophyticus* 15305 resulted in

enhanced adhesion and internalization in both bladder and kidney cell lines. The capacity for *S. saprophyticus* 15305 to invade bladder cells has been previously documented, but the ability of antibiotics to modulate this process has never been explored (Szabados *et al.*, 2008). In addition, this work is the first to demonstrate the *S. saprophyticus* 15305 is capable of invading kidney cells and may represent an important mechanism in urothelial persistence. This is especially relevant in this pathogen, which is suggested to preferentially colonize the kidneys (Kline *et al.*, 2010). Other groups which have assessed the ability of sub-inhibitory antibiotics to enhance *S. aureus* invasiveness found no significant changes (Rasigade *et al.*, 2011). This is despite increases in fibronectin-binding protein expression which improved adherence (Bisognano *et al.*, 2000). The discrepancies between these studies may reflect differences in proposed mechanisms that *S. saprophyticus* 15305 utilizes to invade host cells (Szabados *et al.*, 2008). In the case of *S. aureus*, they primarily rely on their regimen of fibronectin-binding proteins to trigger $\alpha_5\beta_1$ integrin-mediated internalization of non-phagocytic cells (Agerer *et al.*, 2005; Sinha *et al.*, 1999). Although some strains of *S. saprophyticus* express fibronectin and collagen-binding proteins, *S. saprophyticus* 15305 does not (Kuroda *et al.*, 2005). However, staphylococcal autolysins have been implicated as invasins (Hirschhausen *et al.*, 2010), and thus may account for urothelial invasion in *S. saprophyticus* 15305.

Antibiotic treatments had the greatest effect on *S. saprophyticus* 15305 adhesion when urothelial cell surfaces were compared to abiotic surfaces. This led to the speculation that specific adhesin-urothelial interactions were involved and were likely driven by antibiotic-induced expression of adhesion factors. Hemagglutination assays were utilized to determine changes in the relative surface presence of UafA, and suggested ciprofloxacin induced their expression. Bacterial capsules block the interaction of UafA with adhesins (Kuroda *et al*., 2005), but enhanced hemagglutination in the acapsular *S. saprophyticus* C1 strain recapitulated the previous findings. However, differential regulation of capsule cannot be ruled out as antibiotic-dependent modulation in capsular expression has been demonstrated previously in *E. coli* (Lu *et al.*, 2008; Fowler *et al.*, 2009). The influence of ciprofloxacin-induced UafA expression on adherence was confirmed in 5637 bladder cells. Again, ciprofloxacin resulted in enhanced adhesion in the absence of capsule suggesting UafA significantly contributes to this process. In

addition, increased internalization in the acapsular strain treated with ciprofloxacin suggests UafA plays a potential role in *S. saprophyticus* 15305 urothelial invasion. However, this is conjecture and requires further analysis with UafA knock-out strains for confirmation.

Antibiotics were found to also increase the expression of type 1 fimbriae in *E. coli* UTI89 using hemagglutination assays. This adhesin contributes to aggregation and biofilm formation in this organism (Rodrigues and Elimelech, 2009; Schembri *et al.*, 2001). It is interesting that antibiotics from diverse classes should have such a profound impact on adhesin expression, biofilm formation, and aggregation in unrelated Gram-negative and positive uropathogens. As the SOS response has been implicated previously in antibiotic-dependent *E. coli* UTI89 biofilm formation, it was reasonable to postulate a similar role in type 1 fimbrial expression. These experiments revealed that antibiotics were incapable of increasing hemagglutination in the SOS-deficient *E. coli* UTI89 strains, suggesting an important role of this response in type 1 fimbriae regulation during stress. This is supported by previous studies, which have demonstrated involvement of the SOS response in adhesin regulation in *S. aureus*, but not for *E. coli* (Bisognano *et al.*, 2000).

As the SOS regulon controls the expression of dozens of genes in almost all bacteria, it was thought that antibiotic-dependent changes in type 1 fimbriae occurred at the transcriptional level. Type 1 fimbriae regulation is phase variable, and regulated by the *fim* switch element within its operon (Gally *et al*., 1996). However, the analysis of ciprofloxacin exposure on changes in this regulatory element was not revealing. This is not to say that transcriptional modulation does not play a role in antibiotic-dependent type 1 fimbriae expression, but that involvement of the *fim* switch is unlikely. This is especially perplexing considering that mannose-resistant (type 1 fimbriae-independent) hemagglutination was not observed (data not shown), indicating that other fimbrial adhesins, such as P fimbriae, were not expressed following antibiotic treatment (van den Bosch *et al.*, 1980; Hull *et al*., 1981).

4.2.2 Antibiotics concomitantly downregulate motility and upregulate factors critical for adherence

Both *S. saprophyticus* 15305 and *E. coli* UTI89 appear to respond to antibiotic therapy by increasing adhesin expression, adhering to surfaces, and adopting a sessile lifestyle. Reduced metabolic activity imparted by this strategy would certainly benefit both uropathogens to endure times of stress. Adoption of an adherent, inactive lifestyle would require the transition from motile, planktonic states. Demonstrating the suppression of flagellar-mediated motility in *E. coli* UTI89 in response to antibiotics supported this hypothesis. Furthermore, flagella regulation was also assessed in SOS-deficient strains, revealing that activation of this system has a negative influence on motility. This finding agrees with a recent observation of *P. aeruginosa* motility, suggesting SOS is involved in adhesion and biofilm formation in several organisms (Chellappa *et al.*, 2013). Ciprofloxacin, a DNA targeting drug and potent SOS activator, was particularly efficacious in inhibiting wild-type *E. coli* UTI89 swarming, a phenotype which was depressed in the SOS-deficient strains. These findings support the hypothesis that ciprofloxacin concomitantly upregulates adhesin expression and downregulates motility to promote surface adherence. Unexpectedly, bacterial swarming motility in the presence of ciprofloxacin was increased in the *E. coli* UTI89 $lexA_{T355G}$ strain but suppressed in the Δ*recA*. This suggests RecA positively regulates flagellar activity, a finding that has been observed in other strains of *E. coli* (Gómez-Gómez *et al.*, 2007). Although not fully characterized, the mechanism appears to depend on RecA interacting with CheW, a component which governs mechanical control of the chemotaxis system during swarming motility. This finding is further supported, as ciprofloxacin pre-treatment of cells prior to swarming had no effect on flagellar activity.

4.2.3 Transcriptomic analysis provides insight into virulence modulation and antibiotic tolerance mechanisms utilized by Gram-negative and positive uropathogens

The similar phenotypic responses of *S. saprophyticus* 15305 and *E. coli* UTI89 to sub-inhibitory ciprofloxacin was suggestive of a common underlying mechanism explaining changes in virulence trait expression. RNA-sequencing provided a useful high-throughput tool to characterize the global responses during treatment conditions for these uropathogens. Although changes in virulence gene expression were observed for both organisms, the regulatory networks affected by sub-inhibitory ciprofloxacin stress appear to be divergent, despite similar doses relative to the MIC being provided. Specifically, *E. coli* UTI89 committed to the activation of a full-fledged SOS response while *S. saprophyticus* 15305 appeared to rely upon general stress response elements. The nature of cell surface components, including peptidoglycan matrix thickness, membrane abundance/composition and the availability of porins, influences ciprofloxacin penetration and intracellular availability, and might explain different tolerance mechanisms. It is generally accepted that ciprofloxacin is better able to access the intracellular compartment of Gram-negative organisms compared to Gram-positives, enhancing its efficacy in these pathogens (Drlica and Zhao, 1997). Here, *E. coli* UTI89 effectively blocked this route of access by decreasing the availability of various porin transcripts. Interestingly, Berlanga *et al*. (2004) suggested that Gram-positive tolerance to ciprofloxacin may be instead related to the interaction of the drug with its target enzymes rather than diminished permeability. In addition, the decreased replication and transcription rates of *S. saprophyticus* 15305 compared to *E. coli* UTI89 may be conducive to its survival against DNA gyrase and topoisomerase-acting antibiotics while negating SOS activation.

The activation of RecA is associated with phage commitment to the lytic cycle, and evacuation of host cells (Wagner and Waldor, 2002). Both *E. coli* UTI89 and *S. saprophyticus* 15305 initially responded to ciprofloxacin challenge by increasing the abundance of this important DNA repair transcript, but only *E. coli* UTI89 went on to express a number of phage-related SOS response elements. Importantly, the *E. coli* UTI89 genome contains a much larger proportion of phage-coding regions compared to

S. saprophyticus 15305 (as inferred from SEED strip-plots; Figure 3.15). It is possible that activation of RecA in *E. coli* UTI89 triggered the activation of a phage lysis cascade, which in turn contributed to further SOS response commitment in a self-propagating feed-back loop. The Kil-family of proteins translated from phage transcripts observed during ciprofloxacin treatment have been shown to induce the SOS response in *S. enterica* the past (Campoy *et al*., 2006), supporting this novel hypothesis. Counter-intuitively, the phage transition into lytic cycles may enhance the virulence of *E. coli* by permitting the expression of phage-encoded virulence traits during ciprofloxacin therapy. This process has been demonstrated before in *Enterococcus faecalis* and is evolutionarily mutually beneficial to both the host cell and infecting phage (Matos *et al*., 2013). Further analysis using strains cured of their phages may provide insight into the cause-and-effect relationship between onset of the phage lytic cycle and SOS induction.

Sub-inhibitory ciprofloxacin resulted in global suppression of transcripts related to metabolism including amino acid, cofactor, iron, fatty acid, cell division, and carbohydrates in *E. coli* UTI89 after four hours of exposure. This response seems beneficial, as limiting cellular activity decreases the need for transcription and replication, in turn limiting the effect that ciprofloxacin can have on these processes. Remarkably, transcripts related to metabolism and nutrient uptake were found at a higher abundance in *S. saprophyticus* 15305. Specifically, transcripts of enzymes important to glycolysis and the TCA cycle were present at increased quantities following 6 hours of ciprofloxacin exposure. Although the purpose of this change is unknown, TCA induction has been shown to be associated with virulence, survival, and persistence of staphylococci and other pathogens (Begun *et al*., 2005; Sadykov *et al*., 2010). These findings corroborate another transcriptomic-based study which characterized *S. aureus* expression changes in response to inhibitory-doses of ciprofloxacin (Cirz *et al*., 2007). Although a full *S. aureus* SOS response was activated in the study, the authors' results reflect those of this thesis, concluding that an active SOS response is more critical in ameliorating damage caused by DNA-active antibiotics in Gram-negative organisms. Interestingly, these authors did not observe changes in the recruitment of transcripts related to general stress responses. This might suggest that these systems only contribute to the tolerance of DNA stress at levels which are insufficient to induce SOS.

A number of transcripts related to virulence were found to be significantly upregulated in both uropathogens following sub-inhibitory ciprofloxacin exposure. In *E. coli* UTI89, hemagglutination assays suggested that FimH-surface expression might be enhanced by sub-inhibitory antibiotics. The results of the RNA-sequencing analysis confirmed that a putative *fimH*-like element was differentially expressed following ciprofloxacin treatment, indicating antibiotic-dependent hemagglutination is regulated at the transcriptional level. As FimH is critical in the adherence and invasion strategies of UPEC, this finding might indicate a mechanism by which sub-inhibitory antibiotics enhance pathogenesis during UTI. The fimbrial repressor *focX* was also downregulated in response to ciprofloxacin, further highlighting this agent's capacity to mediate fimbrial adhesion. Importantly, expression and cross-talk of the putative *focX*-regulated fimbrial-operon may preclude the transcription of other fimbriae (including type 1), as UPEC generally only express one type at any given time (Simms and Mobley, 2008). This might account for ciprofloxacin's inability to induce type 1 phase switching. Characterization of the ORFs governed by the *focX* regulatory element would provide insight into the mechanisms with which ciprofloxacin induces hemagglutination and biofilm formation in UPEC.

In addition to changes in fimbrial transcripts, the autoaggregative adhesin and invasin *hek* was found to be expressed in the presence of sub-inhibitory ciprofloxacin. This virulence factor has been associated with severe infections including neonatal meningitis and sepsis (Fagan and Smith, 2007; Fagan *et al*., 2008). The *hek* gene is also present in 55% of UPEC isolates (Srinivasan *et al*., 2003), and binds to cells with greater affinity under shear stress suggesting a potential role in urothelial adherence (Fagan and Smith, 2007). Therefore, in addition to FimH, increased expression of this adhesin likely accounts for the ciprofloxacin-dependent aggregation that was observed using TEM. Similarly, the expression of *sulA* during ciprofloxacin treatment is sufficient to account for the filamentation observed using TEM. Transient SulA-mediated filamentation during the IBC phase of pathogenesis is essential for UPEC virulence and QIR formation in murine models of cystitis (Justice *et al*., 2006).

The genome of *S. saprophyticus* 15305 is much less well characterized compared to *E. coli* UTI89, introducing problems in assigning functions to genes that were differentially

regulated in response to sub-inhibitory ciprofloxacin. However, increased surface expression of UafA was posited based on phenotypic assays which revealed a ciprofloxacin-dependent changes in hemagglutination capacity. Unfortunately, RNA-sequencing did not reveal significant changes in the relative abundance of this transcript, suggesting one of the many uncharacterized transcripts upregulated might instead contribute to this observation. Efforts to produce a UafA knock-out strain were not fruitful, but would be essential in ruling out other sheep erythrocyte ligands which could account for UafA-independent hemagglutination. Interestingly, one transcript upregulated by ciprofloxacin encoded a putative bacterial immunoglobulin-like 3-domain (BIG III) protein. Proteins encoding domains belonging to the immunoglobulin-like superfamily are often found in bacterial adhesins (Hamburger *et al.*, 1999). However, similar structures also have calcium-binding potential (Raman *et al.*, 2010). Regardless, these findings provide merit in further characterizing this differentially expressed element in *S. saprophyticus* 15305.

Transcripts encoding enzymes related to exopolysaccharide production and export were among those most highly abundant following ciprofloxacin treatment. Generally, it is thought exopolysaccharide interferes with UafA ligand interactions, a hypothesis which is supported in this work as acapsular strains demonstrate greater adherence to urothelial cells. However, hemagglutination was enhanced in the acapsular strain, suggesting that ciprofloxacin has an effect on either the expression of an uncharacterized adhesin (for example, the upregulated BIG III-containing protein), or that bacterial aggregation caused by this antibiotic influences the appearance of hemagglutination as observed elsewhere (Orndorff *et al.*, 2004). Regardless, exopolysaccharide production is important in resisting opsonophagocytosis (Peterson *et al.*, 1978), mediating mucosal surface adherence, aggregation, and biofilm formation (Stephens *et al.*, 1993; Sutherland, 2001, Li *et al.*, 2005[b]), contributing to persistence during staphylococcal infection (O'Riordan and Lee, 2004), and tolerating antibiotic exposure (Fernebro *et al.*, 2004). Therefore, the increased expression of this virulence factor by ciprofloxacin could certainly enhance the pathogenicity of this uropathogen. In addition, the availability of TCA cycle intermediates are critical for exopolysaccharide production, supporting a purpose for

increasing the activity of this metabolic hub during ciprofloxacin treatment (Sadykov *et al*., 2010).

4.3 SUB-INHIBITORY CIPROFLOXACIN AFFECTS PATHOGENESIS IN A MURINE MODEL OF UTI

Ciprofloxacin-dependent modulation of pathogenesis *S. saprophyticus* 15305 and *E. coli* UTI89 was assessed in a murine model of UTI, developed by Dr. Scott Hultgren's group (Hung *et al*., 2009). Ciprofloxacin was solely utilized in these studies for its widespread administration in urology to treat acute and complicated infections (Krcméry and Naber, 1999), in addition to its capacity to modulate uropathogen virulence. The resistance observed in other antibiotics, such as co-trimoxazole, further warrants investigation of this next-line therapeutic (Eliopoulos and Huovinen, 2001; Talan *et al*., 2000). The murine studies presented within this thesis are the first to demonstrate that antibiotic-dependent *in vitro* augmentation of virulence directly influences *in vivo* pathogenesis.

4.3.1 Ciprofloxacin priming predisposes mice to chronic UTI

Previous results demonstrated that antibiotics are capable of 'priming' organisms for downstream colonization, primarily through the upregulation of adhesins. This approach was utilized for the characterization of both *E. coli* UTI89 and *S. saprophyticus* 15305 pathogenesis in C3H/HeN mice as it removed the parameter of antibiotic-host interactions. However, long-lasting epigenetic effects could also potentially influence downstream virulence in this model (Davidson and Surette, 2008). Type 1 fimbriae phase variation is one such example, but it was not altered by antibiotic therapy here. Infections of C3H/HeN mice with 10^7 cfu of *E. coli* UTI89 resulted in a bimodal infection outcome at 24 hpi, with mice showing signs of either bacterial clearance or maintaining high titers (Hannan *et al*., 2010). This distribution was observed in both control and ciprofloxacin-primed infection groups. However, ciprofloxacin treatment prior to infection shifted the equilibrium towards infection persistence in bladders and kidneys. This effect was most dramatic in the kidneys, with both uropathogens showing no signs of clearance. The different colonization patterns are likely attributable to differences in defence strategies, the foremost of which is superficial umbrella cell exfoliation, a major contributor of pathogen clearance in the bladder (Mulvey *et al*., 2000; Zasloff, 2007).

This finding highlights the shortcomings of this UTI model, with C3H mice demonstrating severe vesicoureteral reflux (VUR) and significant pathogen kidney deposition upon inoculation (Murawski *et al*., 2010; Hung *et al*., 2009). However, there is still merit in using this model for characterization of 'worst-case-scenario' patients, who demonstrate high grade VUR. The results demonstrate that infection severity could be worsened by the application of ciprofloxacin prophylactically, a practice that is widely conducted (Garnica *et al*., 2013; Garin *et al*., 2006; Mattoo, 2009). In addition, *S. saprophyticus* 15305 poorly colonizes the bladder tissues of mice but showed similar infective capacity as *E. coli* UTI89 in the kidneys. The absence of UafA ligand in mice likely accounts for this (King *et al.*, 2011; Matsuoka *et al*., 2011; Kline *et al.*, 2010). It is noteworthy that despite this, ciprofloxacin-priming significantly increased bladder titers, suggesting that other factors might contribute towards virulence. The autolysin Aas has been implicated in fibronectin-binding and thus could contribute to increased adhesive capacity (Hell *et al.*, 1998; von Eiff *et al.*, 2002).

It is established that C3H/HeN mice inoculated with 10^7 cfu of *E. coli* UTI89 presenting with >10^4 cfu/mL at 14 days post-inoculation (dpi) are at very high risk of chronic UTI (Hannan *et al.*, 2010). The increased titers observed following ciprofloxacin priming alluded that these organisms were better equipped to colonize the host compared to untreated counterparts. The host-pathogen interactions during the first 24 hours post-inoculation (hpi) are thought to play a significant role in infection establishment or resolution, suggesting increased titers at this time have some predictive value in determining the severity of UTI (Hannan *et al.*, 2012). The data obtained at 14 dpi with *E. coli* UTI89 validated this hypothesis, with ciprofloxacin-priming prior to inoculation sufficient to increase frequency of chronic cystitis to 100% compared to 60% in untreated groups. While no such data exists describing the infection thresholds of *S. saprophyticus* 15305, it is noteworthy that 100% of mice resolved infection when inoculated with untreated *S. saprophyticus* 15305, while 50% of ciprofloxacin-primed inoculated mice maintained low-grade bladder colonization. Furthermore, bacterial kidney titers were consistently higher in both organisms when primed. The presence of pyelonephritis in the absence of cystitis in this model supports the hypothesis that chronic cystitis and chronic pyelonephritis represent mutually exclusive disease outcomes, governed by different

underlying host-pathogen checkpoints in each organ (Hannan *et al*., 2010; Hopkins *et al.*, 2009).

Though upregulation of adhesins likely contribute to persistence, immune activation is also an important factor in uropathogenesis, with early severe inflammatory responses increasing risk of chronic infection during UPEC UTI (Hannan *et al.*, 2010). Overstimulation and infiltration of immune response elements are thought to promote destruction of the protective urothelium (Ragnarstóttir *et al*., 2010). This might be driven directly by type 1 fimbriae which are known to interact with TLR4 and promote neutrophil infiltration (Mossman *et al*., 2008; Ashkar *et al*., 2008), but it is also possible that upregulation of secreted factors which disrupt the host urothelium contribute significantly to enhanced virulence potential. The potential for antibiotics to promote toxin production in *S. aureus* and *E. coli* has been demonstrated several times, and is associated with increased immunogenicity (Serna IV *et al*., 2008; Dumitrescu *et al*., 2011; Otto *et al*., 2013). However, primed organisms are washed prior to inoculation, presumably removing any secreted factors from the inoculum and requiring continued expression in the mouse urinary tract without antibiotics. In addition, no such toxins exist in *S. saprophyticus* 15305, suggesting this mechanism does not contribute to persistence during bladder infection with ciprofloxacin-primed organisms (Kuroda *et al*., 2005). Rather, it is postulated that exposure of additional surface adhesins, such as UafA and Aas, enhance colonization and internalization efficiency resulting in persistent cystitis. This is supported by *ex vivo* gentamicin protection experiments, which demonstrated the increased invasive capacity of ciprofloxacin-primed *S. saprophyticus* 15305.

4.3.2 Ciprofloxacin priming promotes rapid urothelial invasion and IBC formation

The capacity for ciprofloxacin to promote *E. coli* UTI89 chronic infections is likely dependent on its ability to induce type 1 fimbriae-dependent invasion of urothelial cells. Type 1 fimbriae are critical in internalization and intracellular bacterial community (IBC) formation (Martinez *et al*., 2000; Hadjifrangiskou *et al*., 2012; Wright *et al*., 2007); their expression in response to ciprofloxacin and subsequent chronic infection development is

likely paramount. Increased numbers of IBCs are associated with an increased risk of chronic cystitis and the establishment of quiescent bladder reservoirs, which precede recurrent UTI (Schwartz *et al.*, 2011). The enhanced IBC-forming capacity observed in ciprofloxacin-primed *E. coli* UTI89 indicates that establishment of an intracellular niche is an important step in developing chronic infection in this model. Ciprofloxacin also altered the morphology of IBCs, resulting in a dispersed phenotype compared to untreated controls. The characteristics of these IBCs are reminiscent of those observed during later stages of infection (Justice *et al*., 2004), suggesting ciprofloxacin-priming triggered the upregulation of type 1 fimbriae and early invasion of urothelial tissues. The dynamics of *E. coli* fimbriation are tightly regulated, and production might not otherwise occur immediately upon inoculation into the urinary environment (Schwan, 2011), giving primed organisms an advantage over those left untreated.

Ciprofloxacin-triggered filamentation could potentially contribute to increased urothelial adherence, immune evasion and invasion potential (Justice *et al*., 2006; Justice *et al*., 2008). Filamentation is an important step in *E. coli* flux from IBCs and transfer to neighbouring cells (Rosen *et al*., 2007). Ciprofloxacin-induced changes in *E. coli* UTI89 might hasten the spread to distal tissues before exfoliation can occur, promoting the establishment of quiescent reservoirs in the bladder (Mysorekar and Hultgren, 2006). Observed filamentation at early time-points in ciprofloxacin-treated, but not control IBCs, supports this hypothesis. These processes might further help uropathogens subvert aspects of early host immunity by rapidly gaining access to the intracellular environment and at a higher frequency. Combined, alterations in these pathogenic mechanisms are likely responsible for driving the dynamics of host-pathogen equilibrium in favor of the pathogen and establishment of chronic infection.

4.3.3 Sub-therapeutic ciprofloxacin augments infection severity and recurrence risk in chronically infected and resolved mice

Experiments were carried out to investigate the influence of sub-therapeutic ciprofloxacin on infection severity and recurrence risk. Including this parameter in the experimental

system was critical, as antibiotics including fluoroquinolones are known to modulate aspects of host immunity (Dalhoff and Shalit, 2003). Although the dosing regimens utilized in these experiments are not necessarily reflective of real-world therapeutic approaches, they are useful in studying concomitant changes in both the host and pathogen and therefore contribute to our understanding of antibiotic influence on UPEC pathogenesis *in vivo*.

Two experimental groups were considered to evaluate the influence of ciprofloxacin on: i) the worsening of prognosis in chronically infected mice; and ii) predisposing previously inoculated but 'resolved' mice to clinically significant recurrences. In both cases, sub-therapeutic ciprofloxacin was associated with increased *E. coli* UTI89 urine titers. The effect was compounded over time, suggesting that periods of sub-therapeutic dosing have a cumulative effect on infection severity. Retrospective studies in humans have revealed that repeated exposure to antibiotics are associated with recurrent *C. difficile* infection risk, and found that the response was cumulative over time as observed in this study (Stevens *et al.*, 2011). *C. difficile* infection severity with antibiotics could be due to clearance of commensal organisms from the gastrointestinal tract, decreasing competitive inhibition in this niche (Parkes *et al*., 2009). Although endogenous, non-culturable bacteria have been noted in the urinary tract of healthy females, their role in mucosal defence against infection is not established (Wolfe *et al*., 2012). In addition, spontaneous development of antibiotic resistance could reasonably explain increased urine load with antibiotics, but no changes in susceptibility were noted over the course of the experiment.

The effects that underlie changes in *E. coli* titer with sub-therapeutic ciprofloxacin dosing were concluded to be driven by suppressed host immune capability. Decreased immune potential in mice could reasonably account for *E. coli* UTI89 urine loads increasing in chronically infected subjects. However, the ability of ciprofloxacin to trigger clinically significant recurrences in resolved mice was unexpected, and might occur through several mechanisms. Firstly, the thresholds designating clinically significant infection do not preclude the presence of UPEC. In fact, several mice in these treatment groups did present with bacteriuria, but were considered colonized rather than infected as per clinical standards (Hannan *et al*., 2010; Schwartz *et al*., 2011). Thus, increased titer in these mice

receiving sub-therapeutic ciprofloxacin could be due to a similar phenomenon as observed in chronically infected mice. However, some mice that experienced recurrences had no visible signs of bacteriuria prior to treatment. It is possible that ciprofloxacin triggered re-emergence of *E. coli* from quiescent reservoirs in these apparently sterile mice. The filamentation induction observed in other experiments might cause organisms to flux out from tissues resulting in new infection. However, cross-infection from another mouse within the same cage cannot be ruled out. Interestingly, there were mice that never experienced recurrences with ciprofloxacin therapy, indicating urinary clearance and providing an argument against cross-infection. Regardless, these findings are the first to directly associate inadequate antibiotic therapy with increased infection risk *in vivo*.

4.3.4 Ciprofloxacin modulates aspects of host immunity and is protective against chronic UTI

Ciprofloxacin was found to have an immunomodulatory effect in urothelial tissues, which might account for the changes in bacterial urine titers observed in mice during treatment. The effect was noted in both human bladder cell lines in addition to murine tissues extracted following infection. Low doses of ciprofloxacin were sufficient to depress the release of IL-6 and IL-8 in T24 bladder cells, suggesting that residual levels left over following therapy might predispose to infection later on. The effects were found to be host-cell dependent in 5637 cells, as results in a cell-free system using LPS as an immune-stimulant recapitulated those from the *S. saprophyticus* 15305 infections. Furthermore, the baseline release of cytokines from unstimulated cells did not change with ciprofloxacin addition, rather, the antibiotic appeared to function by preventing secretion when LPS or *S. saprophyticus* 15305 was present. These finding corroborate those of others (Dalfhoff and Shalit, 2003).

IL-6 and IL-8 are both important pro-inflammatory mediators for host cell immunity during UTI. IL-6 is a pleiotropic cytokine whose function includes amplifying signals involved in neutrophil recruitment (Romano *et al*., 1997), while IL-8 is a member of the CXC chemokine family and potent neutrophil chemotactic molecule (Murphy, 1997). Suppression of either cytokine has important implications on neutrophil chemotaxis to

sites of infection (Hedges *et al*., 1994; Hang *et al*., 1999). This was confirmed in mice, which showed significantly depressed PMN infiltrate in urine sediments when treated with sub-therapeutic ciprofloxacin. In addition to IL-6 and IL-8 suppression *in vivo*, the release of several other cytokines during early infection were affected by this antibiotic. The pro-inflammatory mediator IL-1β, produced by activated macrophages, is an important early response element to UPEC infection and was suppressed with ciprofloxacin treatment. Alternatively, the anti-inflammatory cytokine IL-10 was upregulated with infection and ciprofloxacin presence. IL-10 is involved in downregulating co-stimulatory molecules on macrophages, and might be associated with IL-1β suppression (Mosser, 2003; de Waal Malefyt *el al*., 1991). Furthermore, this cytokine is upregulated during UTI and is associated with urothelial protection during acute cystitis (Duell *et al.*, 2011). The capacity for ciprofloxacin to induce one cytokine and suppress another while having no effect in some is perplexing. It might depend on the augmentation of immune populations and their activity within the bladder during infection. Unfortunately, cytological profiles were not assessed, so immune population changes in response to ciprofloxacin are unknown.

A surprising outcome of these studies was the finding that ciprofloxacin dramatically increased the expression of G-CSF. This cytokine increases neutrophil migration from the bone-marrow, and UPEC are known to trigger its upregulation during UTI (Ingersoll *et al.*, 2008). G-CSF presence would be effective in increasing levels of circulating neutrophils, however, the local suppression of chemotactic cytokines in the bladder would diminish the effect at sites of infection. It was not possible to collect sera from these mice and as such ciprofloxacin augmentation of systemic responses are unknown. In addition, G-CSF has immunomodulatory effects on macrophages and attenuates IL-1β production leading to less efficient bacterial clearance which further corroborates our findings (Boneberg and Hartung, 2002; Kim *et al.*, 2006).

The immunomodulatory and urothelial-protective effects of ciprofloxacin were further confirmed using infection models. In this case, decreased neutrophil infiltration and related reduction in urothelial damage is likely, and has been demonstrated in other studies (Ingersoll *et al.*, 2008). Reduction in bladder mass in antibiotic-treated mice is further indicative of suppressed immune cell tissue infiltration. Although certain

cytokine profiles might result in increased infection risk, those associated with ciprofloxacin appear to favour the host. Further studies should explore how these treatments modulate urothelial exfoliation, as cytokines are known to be involved in this process (Mulvey *et al.*, 2001; Bower *et al.*, 2005).

Application of sub-therapeutic ciprofloxacin prior to *E. coli* UTI89 inoculation was sufficient in abrogating the risk of chronic infection. Although no significant changes were observed in organ titer, ciprofloxacin treatment was associated with decreased UPEC abundance. The cytokine profiles generated in this work corroborate other studies which have demonstrated that super-induction of IL-6, IL-8, IL-1β, and G-CSF, result in more severe infection and risk of chronic disease (Ragnarsdóttir *et al*., 2010). Treatment with the anti-inflammatory compound dexamethasone suppresses the release of cytokines during early stages of UPEC infection and provided protection from chronic infection in a similar manner as ciprofloxacin (Sharifian *et al*., 2008; Hannan *et al.*, 2010). These findings suggest that application of antibiotics with immune modulating activities might have an overall protective benefit on the host. However, care should be taken as the risk of developing a resistant infection is significant, and these findings require confirmation in larger population cohorts. Furthermore, although ciprofloxacin is protective in the short-term, the effect on humoral responses has not been characterized. It is possible that the cytokine profiles generated with antibiotics might increase recurrence risk by depressing future adaptive response. Overall, the cytokine profiles detailed in this study demonstrate the dynamic interplay between host defences, which attempt controlled immune recruitment, and *E. coli* pathogenic mechanisms, which aim to super-induce this response.

4.3.5 Ciprofloxacin prophylaxis is not associated with improved outcome and increases the intracellular bladder reservoir in mice

An attempt to demonstrate the effects of ciprofloxacin therapy on UPEC pathogenesis in a therapeutically relevant scenario was accomplished using a murine model of prophylaxis. This model was developed for the purposes of this thesis and had not been

attempted previously. Together, it consisted of an infection event, antibiotic therapy and rest periods, followed by prophylaxis initiation and sacrifice. As mentioned previously, female C57BL/6 mice replaced C3H/HeN mice, which are susceptible to pyelonephritis through VUR. However, the characteristic responses of these mice to UPEC infection are comparable (Hung *et al*., 2009).

Urine titers of mice were tracked over the course of the study. Co-trimoxazole therapy was successful in clearing the bacteriuria in all but two mice, who went on to suffer from chronic infection. During the seven-day rest period, urine titers further demonstrated dynamic changes in bacterial load consistent with clinically significant recurrences. Similar observations have been made by other groups and demonstrate the inadequacy of short-course antibiotic therapy to clear UTI in this model (Blango and Mulvey, 2010; Schilling *et al*., 2002). Admittedly, human patients experiencing such recurrences would restart normal therapy until bacteria were absent from the urine prior to prophylaxis initiation (Dason *et al*., 2011; Kodner *et al.*, 2010). However, the timelines permitted in with this work did not permit such measures to be taken. As such, mice began receiving prophylaxis despite previous recurrences, and in some cases with positive urine cultures. The effect on prophylaxis on bacterial urine loads was found to be negligible. This was even true in the mice that had not cleared their infection with co-trimoxazole. These results reflect human clinical findings, which inevitably question the effectiveness of this management approach (Garin *et al*., 2006; Conway *et al*., 2007; Enzler *et al*., 2011).

Mice were sacrificed and further assessed for augmentation in bladder and kidney *E. coli* UTI89 loads. Although there were no significant changes associated with prophylaxis or vehicle treatment, there was trend towards higher titers when ciprofloxacin was provided. In some cases, these changes might have clinical significance. For example, the bladders from all animals receiving prophylaxis maintained some bacterial load, while those in the control group showed signs of resolution. Only one animal in the control group maintained kidney titers while six in the prophylaxis remained colonized. The most important finding from these studies was in revealing the propensity for prophylaxis to enhance UPEC tissue invasion in bladders. These results mirror clinical observations, in that while prophylaxis might assist in decreasing UTI symptoms, they inexorably do not alter the long-term risk of recurrence, and may in fact promote future episodes by

establishing intracellular reservoirs. The presence of intracellular UPEC has been associated with recurrence risk both in humans (Garofalo *et al.*, 2007) and mice (Schwartz *et al.*, 2011) in the past. Together, the data presented in this thesis provide compelling evidence that low-dose antibiotics are capable of enhancing virulence and pathogenesis during UTI.

4.4 PERSISTER CELLS CONTRIBUTE TO RECURRENT UTI BY IMPROVING SURVIVAL DURING ANTIBIOTIC THERAPY

The results from the prophylaxis model demonstrate that antibiotics, even when applied at therapeutic levels, are incapable of sterilizing the urinary tracts of mice. These findings have been noted in several other studies, using a wide variety of agents and different pathogens (Blango and Mulvey, 2010). Although the presence of organisms in protective intracellular niches could account for these findings, the observation of organisms in the urine and lumen of mice during prophylaxis suggest other mechanisms must also contribute. Antibiotic tolerant persister cells were thought to play an important role in survival, and were characterized in this thesis in the context of recurrent UTI.

4.4.1 The nature of persistence and MDT in uropathogens

Two population-based approaches were used to characterize the persister fractions of representative uropathogens. The observation of variations in persister fraction sizes between Type I stationary and Type II exponential sub-populations indicate that global dormancy cannot solely account for MDT. Rather, it is proposed that persisters can be classified based on the metabolic activity of distinct cellular processes such as peptidoglycan cross-linking, transcription and translation, with each demonstrating various degrees of activity and influencing a persister's susceptibility to antibiotics. In this way, MDT is a function of whole populations, but not individual persisters, which are heterogeneous in regards to their antibiotic sensitivity and tolerance. Furthermore, antibiotic-induced tolerance is not dependent on onset of global dormancy, as cross-tolerance results suggest that an individual persister's susceptibility to different agents is contingent on its mechanism of onset. These results reflect other phenotypic observations, such as filamentation, which occur in only a subset of the overall populations. An organism's commitment to certain phenotypes may be stochastic in nature, perhaps due to antibiotic-dependent changes to noise propagation in gene networks, or augmentation in organelle and molecule partitioning during cell division (Pedraza and Oudenaarden, 2005; Huh and Paulsson, 2011).

Generally, most antibiotics appear to primarily influence their cognate target, increasing tolerance against further challenge with the same agent rather than resulting in global down-regulation of overall cellular activity as hypothesized (Grønlund and Gerdes, 1999). However, there are notable exceptions, including that ampicillin pre-treatment results in heightened MDT against all agents in *S. saprophyticus* 15305. The ability of this drug to halt replication in an SOS-dependent manner and induce β-lactam tolerance has been demonstrated in *E. coli*, but is perhaps more significant in Gram-positive bacteria (Maisonneuve *et al*., 2011, Miller *et al*., 2004). Agents affecting transcription and translation may impart more broad-spectrum tolerance by influencing downstream processes and therefore the activity of other potential drug targets in a similar manner. SOS-deficient UPEC strains were deficient in antibiotic-induced persister formation against all agents tested. Surprisingly, gentamicin induction was also affected despite its reported inability to induce SOS responses in *E. coli* (Baharoglu *et al*., 2013). This might reflect a novel SOS-dependent mechanism of persister induction. Considering the heterogeneity in Gram-negative and Gram-positive responses, various antibiotics may demonstrate different persister-inducing potentials against different organisms. If true, this hypothesis may have predictive clinical value in the prescribing of antibiotics, which effectively ameliorate disease without unnecessarily enriching persister fractions.

The activation of SOS systems and release of Lon protease mediates antitoxin degradation resulting in intracellular toxin build-up from numerous TA modules and persister formation (Maisonneuve *et al*., 2011). These modules also appear to play a role in dormancy maintenance, with sequestration of the toxin by its constitutively expressed cognate antitoxin occurring to release cells from persistence (Hansen *et al*., 2008). As antibiotics can influence SOS activity, their effect on persister stability and the dynamics of resuscitation were investigated. Although antibiotic pre-treatments influence the expression of these genes and result in dormancy onset, their presence does not seem to alter the general kinetics of resuscitation. However, the dichotomy in resuscitation rates between *E. coli* CFT073 and *S. saprophyticus* 15305 further suggests that mechanisms of persister maintenance can vary between organisms. The potential application to chronic infections is unclear and requires further exploration, as characterization of these traits

might have the potential to enhance treatment strategies by outlining periods of vulnerability during infection (Gefen *et al*., 2008).

Enumeration of the Type I and Type II persister fractions of UPEC isolates revealed heterogeneity in both sub-populations' responses to antibiotics. Ciprofloxacin significantly reduced survival in stationary phase cultures compared to ampicillin and gentamicin treatments which were largely ineffective against both SSR and AI isolates, as demonstrated in other strains tested. The comparatively enhanced efficacy observed for the DNA gyrase inhibitor ciprofloxacin suggests significant activity in persisters. This may indicate that transcription and replication actively occur in persisters and that they are sensitive to changes affecting these processes (Ramage *et al*., 2009). This hypothesis is further confirmed through the observation that SOS-deficient mutants are severely inhibited when challenged with ciprofloxacin, while remaining refractory to ampicillin and gentamicin, agents that do not directly damage DNA. In addition to revealing DNA gyrase activity in persisters, this result also suggests that an active SOS response is required to abrogate ciprofloxacin-induced DNA damage (especially with respect to RecA-dependent double-strand break repair), which may be critical for fluoroquinolone tolerance in UPEC. The concurrent inhibition of the SOS response (such as with the application of RecA inhibitor N^6-(1-Napthyl)-ADP) along with the application of fluoroquinolone antibiotics may be an effective means to clear persister-dependent chronic infections (Lee *et al*., 2005).

4.4.2 UPEC isolated from patients with same-strain recurrences have larger persister fractions than those pathogens cleared with normal therapy

Type II persisters were induced by antibiotics at a high frequency in SSR compared to AI isolates, suggesting these traits may be subject to selection and enriched over the course of recurrent UTI and prophylaxis. Selection of genes influencing persistence has been previously demonstrated, where overproduction of the persister-inducing molecule indole corresponded with increased antibiotic levels over time and subsequent MDT (Vega *et al*., 2012). In addition, Lewis' group observed that *P. aeruginosa* persister fractions

increased over a 96 month period in an individual suffering with cystic fibrosis and undergoing antibiotic therapy (Mulcahy *et al*., 2010). These results are intriguing as antibiotics often increase the incidence of mutation within organisms, thereby increasing the frequency with which organisms gain persister traits during long-term prophylactic therapy (Kohanski *et al*., 2010). This observation may have severe clinical ramifications in suggesting that a patient's history of antibiotic use may increase their risk of going on to suffer from recurrent infections. In some cases, SSR organisms not only survived lethal antibiotic dosing, but actually continued replicating in the presence of the agents despite their susceptibility. This resistance-like phenotype has been observed previously in susceptible Type II persister fractions but is often unreported. Notably, Balaban's group (2004) demonstrated that *E. coli* persisters with mutations in the TA *hipQ* gene could continue limited growth when exposed to lethal ampicillin, corroborating our results. Other groups have noted periods of slight replication following lethal antibiotic dosing (Keren *et al*., 2011, Wiuff *et al*., 2005). Wiuff *et al*. (2005) concluded this could not be explained by degeneration of the antibiotic in the growth media over time alone. Maisonneuve *et al.* (2013) recently showed that slow growing variants within an exponentially growing *E. coli* population demonstrate MDT in a (p)ppGpp-dependent manner. Admittedly, it is possible that the observed limited replication of persisters is a result of the categorical susceptibility cut-offs provided by the Kirby-Bauer method of testing. Although strains appeared as susceptible during this routine test, they were often highly tolerant with low ZOIs (nearly intermediately to resistant susceptibility). This is of clinical concern as antibiograms may inaccurately designate intermediately susceptible organisms as fully susceptible. However, the observed growth of persisters in this and other studies is supportive that these cells maintain at least some metabolic activity.

Further analysis and comparisons using cross-tolerance illustrated that SSR isolates have a greater capacity to form persisters following antibiotic pre-treatment than AI strains. However, ampicillin often failed to induce persistence in clinical isolates tested, with pre-treatment resulting in greater susceptibility especially following gentamicin challenge. This finding supports the notion that persister cells demonstrate limited replication, as ampicillin was capable of corrupting the still moderately active transpeptidase enzyme. The resulting leakiness likely improved the uptake of gentamicin, which in turn enhanced

its efficacy against the still active ribosome once available intracellularly (Maisonneuve *et al*., 2011). This is supported by Collins' group who demonstrated that increasing gentamicin uptake resulted in aminoglycoside sensitivity in *E. coli* (Allison *et al*., 2011). The observation that persister cells do not demonstrate global metabolic dormancy suggests that some combinatorial antibiotic therapies may be effective in treating persister-related chronic infections. Larger, high-throughput screens are recommended to validate this hypothesis.

In summary, persisters are differentially induced at sub-inhibitory levels by ampicillin and gentamicin in addition to ciprofloxacin in both Gram-negative and Gram-positive bacteria. Type I persister fraction analysis indicates that global metabolic dormancy is not solely responsible for MDT. Rather, the metabolic activity of individual targets dictates overall bacterial responses to agents applied, with some antibiotics imparting a greater inhibitive effect on multiple cellular targets than others. This is supported in SOS-deficient strains, which are unable to specifically tolerate ciprofloxacin. Persister traits are enriched in organisms derived from patients with a history of antibiotic therapy. As low-dose suppressive antibiotic therapy and antibiotic prophylaxis are commonly utilized regimens in clinical medicine, they may in fact be playing a role in the development of MDT. The findings in this thesis raise concern that the incidence of chronic and recurrent bacterial infections may increase with the use of antibiotics for prophylaxis, corroborating a recent theory that modern recurrent UTI frequency correlates with the widespread use of antibiotics (Nickel, 2005). A greater understanding of these unique bacterial physiological states will be essential to improve management of these common debilitating infections. Specifically, treatment should focus not on the 'waking' of persisters, but rather on the characterization of cellular targets that are active and thus susceptible to antibiotic corruption. On a broader scale, the fact that antibiotics can induce persister states emphasizes the need to avoid where possible, exposure to low-dose concentrations of these agents, either as prophylactic agents or through environmental contamination.

4.5 RE-EVALUATING ANTIBIOTIC APPROACHES IN THE CLINICAL SETTING

Having delineated the off-target effects of some antibiotics we can now begin to approach therapy with novel strategies aimed at improving patient recovery while reducing the risk of complication. Simply changing the manner in which antibiotics are delivered may be a quick and viable approach to this problem (Craig, 1998). This is no easy task as organism responses can be mixed, with certain factors induced and others suppressed depending on the agent applied and strain challenged (Davies *et al.*, 2006). Combinatorial, suppressive therapies may be the best approach to ensure lethal antibiotic delivery is achieved at sites of infection. Indeed, some antibiotic combinations have been proven to synergistically inhibit the expression of toxin genes (Subrt *et al.*, 2011) and prevent resistance development (Zhanel *et al.*, 2006; Berti *et al.*, 2012). However, effectiveness is questionable as organisms not cleared during initial treatment phases can go on to develop multidrug resistance much quicker than had the therapy been single regimen (Pena-Miller *et al.*, 2013). Similarly, hospital-wide antibiotic cycling strategies are currently not recommended as they promote the rapid accumulation of resistance traits to multiple drug classes (Masterton *et al.*, 2010). Rather, antibiotic rotation at the patient level (referred to as heterogeneity mixing or Periodic Antibiotic Monitoring and Supervision) is a more structured and specific approach to preventing resistance development and spread, but more validation is needed (Masterton *et al.*, 2010).

A simpler approach to curtail resistance may be to adjust the timing of dosing such that a 'mutation prevention concentration' is achieved (Cantón and Morosini, 2011). This involves minimizing the 'mutant selection window' by ensuring sub-inhibitory levels are avoided throughout the course of therapy. Thus, antibiotics which are delivered to therapeutic sites at high concentrations and then rapidly cleared would be optimal to limit onset of resistance and virulence gene expression (Krasniqi *et al.*, 2012). In some cases, antibiotic therapy may be avoided all together. For example, it is well known that some infections can self-resolve or more quickly resolve with augmentation of probiotics (Reid *et al.*, 2010). One of the largest contributors to antibiotic prescriptions worldwide is attributed to UTI, which also represents a major reservoir for resistance (Foxman, 2010).

Bleidorn *et al.* (2010) demonstrated that ibuprofen was just as effective as the antibiotic ciprofloxacin in ameliorating symptoms associated with uncomplicated UTI. This finding suggests that some infections may be readily cleared by host immune responses, while physicians can assist in minimizing patient symptoms in a 'watchful-waiting' approach, providing antibiotics only when it is absolutely required. Some temporary medical devices, such as ureteral stents that leach immune-modulating components may help reduce symptoms and the need for additional medication (Mendez-Probst *et al.*, 2012). These approaches are worthy of more rigorous pursuit, as antibiotics should be reserved for when they are absolutely required. The increasing frequency of non-reverting resistant strains which do not demonstrate any measurable fitness cost support this (Sundqvist *et al.*, 2010). Once established these strains are here to stay and hamper current antimicrobial stewardship programs, which are designed to curtail resistance through negative selection after it has arisen.

Employing novel agents which prevent the activation of general stress response elements, specifically those targeting the SOS system may prove efficacious in curtailing hypervirulence, resistance and mutator phenotypes. RecA, a protein important to activation of SOS, has shown to be a promising therapeutic target when suppressed with inhibiting agents given in conjunction with antibiotics (Lee *et al.,* 2005; Sexton *et al.,* 2010; Wigle *et al.*, 2009; Do Thi *et al.*, 2011). An array of RecA inhibitors have already demonstrated effectiveness in curtailing the rate of spontaneous resistant mutants. In addition, these agents may also be useful in limiting the antibiotic-dependent production of persister cells and virulence determinants which occur in an SOS response dependent manner. Similarly, SOS suppression by a novel, engineered bacteriophage served as an adjuvant to enhance killing by quinolone antibiotics (Lu and Collins, 2009). This approach was effective *in vivo*, successfully killing resistant organisms, persister cells, and biofilm associated cells. Avoiding activation of stress responses altogether may be another appealing alternative for managing infection. Compounds like mannosides, pilicides and curlicides may accomplish this and function by interfering with pathogen-host binding. Promise in preventing and treating UTI has already been demonstrated (Guiton *et al.*, 2012; Cegelski *et al.*, 2009). High-throughput screening methods to find other 'non-antibiotic drugs' capable of potentiating the activity of widely used antibiotics

have been successful to some degree (Ejim *et al.*, 2011). The advantage of this approach is that co-therapies are more selective than standard therapies in preferentially targeting pathogenic species while limiting destruction of the host microbiota.

Effective treatment strategies will also need to eliminate or reduce persister fractions, although it seems unlikely that any single antibiotic therapy could accomplish this (Allison *et al.*, 2011). Thus, novel treatment strategies must be conceived to control chronic, persister-dependent infections. One such strategy involves activating a surface protease using acyldepsipeptide (ADEP4), which leads to self-digestion and death of pathogens when used in combination with antibiotics (Conlon *et al.*, 2013). Another method used mannitol to induce activation of various metabolic pathways in *E. coli* and *S. aureus* persisters, potentiating the uptake of gentamicin and promoting ribosome corruption (Allison *et al.*, 2011). The issue will now be to develop such products, given the challenges, costs and longevity of the drug development process. However, if these strategies can be applied clinically they will have a great impact on not only eliminating chronic infections, but also in decreasing the development and spread of resistance.

4.6 CONCLUSIONS

Several conclusions can be drawn from the findings presented within this thesis, which should be considered in the wider context for their potential implications for prophylactic and therapeutic antibiotic use:

1) Sub-inhibitory antibiotics from a broad range of classes are capable of modulating the virulence potential of various uropathogens. This finding is suggestive of the ubiquitous nature of the responses observed, with regard to antibiotics applied, and organisms challenged. Although a number of phenotypes may be induced with antibiotics, the onset of a sessile lifestyle due to suppressed motility and increased adhesin and exopolysaccharide expression is prominent.

2) Induction of the SOS response is in part responsible for the phenotypic changes incurred as a result of sub-inhibitory antibiotic treatment. The changes following antibiotic dosing were abolished in the SOS-deficient *E. coli* UTI89 strains challenged. Although the influence of SOS was not explored in *S. saprophyticus* 15305, other groups have found this system to impart phenotypic changes in other staphylococci with antibiotic exposure. However, transcriptomic analysis reveals that general stress responses are also involved in sub-inhibitory antibiotic tolerance in this uropathogen.

3) Sub-inhibitory antibiotic-induced changes of virulence gene expression *in vitro* reflect pathogenic changes *in vivo* using a murine model of UTI. Ciprofloxacin-priming was sufficient to promote the establishment of chronic reservoirs in the bladders and kidney of mice infected, in both *S. saprophyticus* 15305 and *E. coli* UTI89. These changes likely depended on the rapid invasion of urothelial tissues in the first 24 hours following inoculation, and shifts in host-pathogen checkpoint equilibrium in favour of the pathogen and establishment of chronic infection.

4) Ciprofloxacin exhibits immunomodulatory effects in both mice and human tissues, and is protective against the establishment of chronic UPEC infection. These changes likely depend on cytokine profile augmentation in urothelial tissues, which suppress neutrophil recruitment and inflammation at sites of infection.

5) Ciprofloxacin prophylaxis is not recommended for recurrent infections. Mice receiving prophylaxis were not associated with significant changes in UPEC urine titer compared to those receiving vehicle. Furthermore, animals receiving prophylaxis presented with greater intracellular bladder reservoirs than the control group, which might predispose to future recurrences.

6) The persistence of bacteria in the urinary tracts of patients suffering from recurrent infections is likely due to the presence of MDT persister cells. Antibiotics are capable of triggering this tolerant state, and might select for organisms with larger persister fractions during prolonged therapy. Furthermore, persister cells do not exhibit the properties required for complete metabolic dormancy, suggesting that anti-persister drug development should focus not on the 'waking' of these variants, but on potentiating the activity of currently available antibiotics, such as through the use of RecA inhibitors.

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Appendices

Appendix A: Copyright from *Journal of Endourology*

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Title: Third Prize: Effects of Subinhibitory Concentrations of Ciprofloxacin on Staphylococcus Saprophyticus Adherence and Virulence in Urinary Tract Infections

Author: Petar Erdeljan, Kyle W. MacDonald, Lee W. Goneau et al.

Publication: Journal of Endourology

Publisher: Mary Ann Liebert, Inc.

Date: Jan 1, 2012

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Appendix B: Copyright from *Antimicrobial Agents and Chemotherapy*

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AMERICAN SOCIETY FOR MICROBIOLOGY

Title: Selective Target Inactivation Rather than Global Metabolic Dormancy Causes Antibiotic Tolerance in Uropathogens

Author: Lee W. Goneau, Nigel S. Yeoh, Kyle W. MacDonald et al.

Publication: Antimicrobial Agents and Chemotherapy

Publisher: American Society for Microbiology

Date: Apr 1, 0001

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CURRICULUM VITAE

LEE WILLIAM GONEAU

Department of Microbiology and Immunology,
Schulich School of Medicine and Dentistry, Western University, Canada
Lawson Health Research Institute (LHRI)

EDUCATION

03/2011 – Present **Graduate Studies - Doctorate of Philosophy**
Department of Microbiology and Immunology,
Western University, Canada
Supervisor: Dr. Gregor Reid

03/2013-07/2013 **Graduate Studies - Doctorate of Philosophy (exchange)**
Department of Molecular Microbiology
Washington University School of Medicine, St. Louis, USA
Supervisor: Dr. Scott Hultgren

09/2009-03/2011 **Graduate Studies - Master of Science (transfer)**
Department of Microbiology and Immunology,
University of Western Ontario, Canada
(Successfully transferred to PhD program 03/2011)
Supervisor: Dr. Peter Cadieux

09/2005 – 04/2009 **Undergraduate - HBSc**
Departments of Biochemistry and Biology,
University of Western Ontario, Canada

09/2000 – 06/2004, 09/2004 – 06/2005 **Ontario Secondary School Diploma**
Central Secondary School,
Clarke Road Secondary School,
London, Canada

RESEARCH AND COLLABORATIVE EXPERIENCE

06/2013 – Present Research Assistant
London Health Sciences Center, London, ON, Canada
Department of Pathology and Laboratory Medicine
Supervisor: Dr. Johan Delport

03/2013 – 07/2013 Visiting Scientist
Washington University School of Medicine, St. Louis, MO, USA
Department of Molecular Microbiology
Supervisor: Dr. Scott J Hultgren
Funded by CGS - Michael Smith Foreign Study Supplement

01/2011 – 09/2013 Microbiology Consultation
Kensey Nash Corporation, Madison, WI, USA
Project Leaders: Dr. Jeffrey Dalsin, Dr. Peter Cadieux, Dr. Hassan Razvi, Dr. Jeremy Burton

09/2009 – 04/2012 Microbiology Consultation
University of Windsor, Windsor, ON, Canada
Department of Civil and Environmental Engineering
Project Leaders: Dr. Rupp Carriveau, Dr. Peter Cadieux

05/2007 – 09/2009 Research Assistant
Urology Research Laboratory, LHRI
Supervisor: Dr. Peter Cadieux

AFFILLIATIONS

09/2013 – Present The Canadian Society of Microbiologists

TEACHING AND SUPERVISORY EXPERIENCE

Research Supervisor – Endourology Fellow and Resident Laboratory Training Program

- 01/2011-Present – Dr. Varunkumar Bathini
- 04/2012-06/2013 – Dr. Nader Fahmy
- 04/2011-06/2012 – Dr. Andrew Fuller
- 07/2010-06/2011 – Dr. Petar Erdeljan
- 07/2010-06/2011 – Dr. Alfonso Carreno
- 07/2009-06/2010 – Dr. Carlos Mendez-Probst
- 07/2007-06/2008 – Dr. Geoffrey Wignall

Teaching Assistantship – Biology of Prokaryotes (2100a), Department of Microbiology and Immunology – UWO

- 09/2012-12/2013 - **nominated for institutional TA awards**
- 09/2011-12/2011 - **nominated for institutional TA awards**
- 09/2010-12/2010

Chinese Scholarship Exchange Program

- 09/2012-Present - Dr. Yige Bao (West China Hospital of Sichuan University)
- 09/2011-09/2012 - Dr. Luo Yang (West China Hospital of Sichuan University)

Partners in Experiential Learning Program

- 09/2011-01/2012 – Madhavi Gupta (Lucas Secondary School)

Sanofi-Aventis Biotalent Challenge and London District Science and Technology Fair

- 01/2010-05/2010 - Amy Hanif (Westminster Secondary School)

MSc Candidate (Orthodontics), Department of Dentistry - UWO

- 04/2008-04/2009 - Dr. Manisha Jindal

Schulich Summer Dental Awards Program, Department of Dentistry - UWO

- 06/2012-09/2012 - Ryan Lum-Tai
- 06/2011-09/2011 – Michael Lung
- 06/2010-09/2010 - Eva Adam

TEACHING AND SUPERVISORY EXPERIENCE (CONTINUED)

Fourth Year Thesis Project Student (4970E) Supervisor, Department of Microbiology and Immunology - UWO

- 09/2013-Present – Kirstie Cockwell
- 09/2011-04/2012 – Amanda Ruprecht
- 09/2010-04/2011 – Kyle MacDonald
- 09/2009-04/2010 – Elizabeth Montgomery
- 09/2008-04/2009 – Ola Ismail

Schulich Research Opportunities Program, Department of Medicine – UWO

- 06/2012-09/2012 – Garret Mosey
- 06/2011-09/2011 – Melissa Huynh
- 06/2009-09/2009 – Brad Rowe

Urology Research Group Mentorship Initiative

- 09/2011-04/2012 - Nicole Zeit (Faculty of Law, Western University)

SCHOLARSHIPS, AWARDS AND HONOURS

2012-2015 **Canadian Institutes of Health Research Frederick Banting and Charles Best Canada Graduate Scholarship**

- National Award ($105,000 over 3 years)

2013 **Canadian Graduate Scholarships - Michael Smith Foreign Study Supplement**

- National Award ($6,000)
- Funding for international research, Laboratory of Dr. Scott Hultgren, Washington University School of Medicine, St Louis, MO, USA

2012-2013 **Ontario Graduate Scholarship**

- Ontario Government Award ($15,000)
- **Award Declined -** Accepted Canadian Institutes of Health Research Frederick Banting and Charles Best Canada Graduate Scholarship

2012-2013 **Teaching Assistant Award Nominee**

2012-2013 **Western Graduate Research Scholarship**

- University of Western Ontario Internal Award - Tuition Scholarship ($7,816)

SCHOLARSHIPS, AWARDS AND HONOURS (CONTINUED)

11/2012 **Cedar Lane and Novus Biologicals Oral Presentation Award**
- Infection and Immunity Research Forum - Best Oral Presentation ($500)

2011-2013 **Teaching Assistant Award Nominee**

2011-2012 **Queen Elizabeth II Graduate Scholarship in Science/Technology**
- Ontario Government Award ($15,000)

2011 **Endourology Fellowship Manuscript Award (Basic Science Division)**
- International Endourological Society - Third Prize ($750) (Co-author)

2011-2012 **Western Graduate Research Scholarship**
- University of Western Ontario Internal Award - Tuition Scholarship ($8,508)

2010-2011 **Lawson Health Research Institute Studentship Award**
- LHRI Internal Award ($9,000)

09/2010 **Travel Award**
- Department of Microbiology and Immunology, UWO ($1,000)
- Support for World Congress of Endourology Annual Meeting

2010-2011 **Schulich Scholarship for Medical Research**
- University of Western Ontario Internal Award ($2,270)

2010-2011 **Schulich Graduate Enhancement Scholarship**
- University of Western Ontario Internal Award ($5,000)

2010-2011 **Western Graduate Research Scholarship**
- University of Western Ontario Internal Award - Tuition Scholarship ($5,118)

04/2010 **Research Western Imagination Prize (Co-supervisor)**
- London District Science and Technology Fair ($200)

2009-2010 **Schulich Graduate Enhancement Scholarship**
- University of Western Ontario Internal Award ($5,000)

SCHOLARSHIPS, AWARDS AND HONOURS (CONTINUED)

2009-2010 **Western Graduate Research Scholarship**
- University of Western Ontario Internal Award - Tuition Scholarship ($7,081)

10/2007 **World Congress of Endourology Poster Award (Second Prize)**
- International Endourological Society (Co-author) ($500)

2005 **Ontario Scholar Award**
- University of Western Ontario Internal Award ($1,500)

PEER REVIEWED PUBLICATIONS

1. **Goneau LW**, Yeoh NS, MacDonald KW, Cadieux PA, Burton JP, Razvi H, Reid G. (2014) Selective target inactivation rather than global metabolic dormancy causes antibiotic tolerance in uropathogens. *Antimicrobial Agents and Chemotherapy* (doi:10.1128/AAC.02552-13). Featured as a Current Topic in ASM's *Microbe Magazine* - "Two Persisters: One Hides, Another Shuts Down Specific Targets".

2. Fahmy N, Woo M, Alameldin M, Lee JK, MacDonald K, **Goneau LW**, Cadieux P, Burton P, Pautler S. (2013) Endogenous biotin expression in renal and testicular tumors and literature review. *Canadian Urological Association Journal* (Accepted December 2013).

3. Fahmy N, Woo M, Alameldin M, MacDonald K, **Goneau LW**, Cadieux P. (2013) Ochratoxin A is not detectable in renal and testicular tumors. *Canadian Urological Association Journal* (Accepted August 2013).

4. Bevan T, **Goneau LW**, Cadieux PA, Razvi H, Carriveau R. (2012) Numerical simulation of peristaltic urine flow in a stented ureter. *American Journal of Biomedical Sciences* 4(3):233-48.

5. Mendez-Probst CE, **Goneau LW**, MacDonald KW, Nott L, Elwood CN, Lange D, Chew B, Denstedt JD, Cadieux PA. (2012) The use of triclosan eluting ureteral stents effectively reduces ureteral stent symptoms: a prospective randomized trial. *British Journal of Urology International* 110(5):749-54.

PEER REVIEWED PUBLICATIONS (CONTINUED)

6. Erdeljan P, MacDonald KW, **Goneau LW**, Bevan T, Carriveau R, Razvi H, Denstedt JD, Cadieux PA. (2011) Effects of subinhibitory concentrations of ciprofloxacin on *Staphylococcus saprophyticus* adherence and virulence in urinary tract infections. *Journal of Endourology* 26(1):32-7. Third Prize (Basic Sciences Division).

7. Cadieux PA, Chew BH, Nott L, Seney S, Elwood CN, Wignall G, **Goneau LW**, Denstedt JD. (2009) Use of triclosan-eluting ureteral stents in patients with long-term stents. *Journal of Endourology* 23(7):1187-1194.

8. Wignall GR, **Goneau LW**, Chew BH, Denstedt JD, Cadieux PA. (2008) The effects of triclosan on uropathogen susceptibility to clinically-relevant antibiotics. *Journal of Endourology* 22(10):2349-2356.

SUBMITTED OR IN PREPARATION MANUSCRIPTS

1. **Goneau LW**, Jindal M, MacDonald K, Gupta M, Burton J, Hatibovic-Koffman S, Cadieux P. (2014) Novel Management Strategies for the Control of *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans* Biofilms.

2. **Goneau LW,** Hannan TJ, Gloor GB, MacPhee RA, Scwartz DJ, Razvi H, Burton JP, Hultgren SJ, Reid G. (2014) Inadequate antibiotic therapy enhances the risk of chronic infection in a murine model of UTI.

3. **Goneau LW,** Delport J, Razvi H, Burton JP, Reid G. (2014) The dangers of inappropriate antibiotic therapy - issues beyond resistance.

PUBLISHED COLLECTIVE WORKS

1. **Goneau LW**, MacDonald KW, Huynh MJ, Whiteside SA, Yang L, Cadieux PA. (2012) Hormetic Effects of Antibiotics on Bacteria and their Potential Role in Recurrent Urinary Tract Infection. *Urotoday.com* (Invited Beyond the Abstract Commentary - Effects of Subinhibitory Concentrations of Ciprofloxacin on *Staphylococcus saprophyticus* Adherence and Virulence in Urinary Tract Infections), (ISSN 1939-4810 - http://www.urotoday.com/infections-1165)

PUBLISHED PEER REVIEWED ABSTRACTS

1. **Goneau LW*,*** Yeoh N, Cadieux P, Burton J, Hassan R, Reid G. (2013) Management of Recurrent Urinary Tract Infections: Something's Wrong with Diagnosis and Treatment. *Canadian Urological Association Journal*, 7(5-6Suppl2):S86.

2. **Goneau LW**, MacDonald K, Razvi H, Cadieux PA. (2012) Sub-Minimal Inhibitory Concentrations of Antimicrobials Prime *Staphylococcus saprophyticus* for Survival Within the Urinary Tract During Prophylaxis. *Canadian Urological Association Journal*, 6(4):319:P98.

3. Nader F, Woo M, MacDonald K, **Goneau LW**, Cadieux PA, Pautler S. (2012) Investigating Ochratoxin A and Biotin Levels in Patients with Renal Carcinoma. *Canadian Urological Association Journal*, 6(4):317:P94.

4. Bathini V, Fuller A, **Goneau LW**, MacDonald K, Razvi H, Cadieux PA. (2012) Urinary Device Attachment of *Staphylococcus saprophyticus* in the Presence of Sub-Minimal Inhibitory Antimicrobial Concentrations. *Canadian Urological Association Journal*, 6(4):317:P92.

5. Fahmy N, Fuller A, **Goneau LW**, MacDonald K, Erdeljan P, Bathini V, Razvi H, Cadieux P. (2012) Sub-inhibitory Antibiotic Concentrations Negatively Affects Both the Uropathogen *Staphylococcus saprophyticus* and Host Immune Responses. *Canadian Urological Association Journal*, 6(3 Suppl1): S92.

6. Fuller A, **Goneau LW**, MacDonald K, Erdeljan, P, Bathini V, Razvi H, Cadieux PA. (2011) Sub-Inhibitory Antibiotic Concentrations Enhance Surface Attachment, Survival and Host Immune Evasion in the Uropathogen *Staphylococcus saprophyticus*. *Canadian Urological Association Journal,* 5(5 Suppl 3): S181. Selected as highlight at the NSAUA 2011 Annual Meeting, New Orleans, USA.

7. **Goneau LW**, Mendez-Probst C, Razvi H, Cadieux PA. (2010) Effects of Antimicrobials on Biofilm Formation in *Staphylococcus saprophyticus*. *Journal of Endourology* 24(S1).

8. Cadieux PA, Chew BH, **Goneau LW**, Lange D, Vanjecek M, McCormick JK, Denstedt JD. (2009) Recombinant oxalate decarboxylase YvrK degrades oxalate in a dose- and time-dependent manner. *Journal of Urology* 181(4):659-660.

PUBLISHED PEER REVIEWED ABSTRACTS (CONTINUED)

9. Wignall G, **Goneau LW**, Chew BH, Denstedt JD, Cadieux PA. (2008) Triclosan enhances antibiotic susceptibility in several common uropathogens. *Journal of Urology* 179(4 Supp):84.

10. Wignall G, Chew BH, Denstedt JD, **Goneau LW**, Cadieux PA. (2007) Triclosan renders several uropathogens increasingly susceptible to relevant antibiotics. *Journal of Endourology* 21 (Supp 1):BR1-17. Selected as a highlight at the WCE 2007 Annual Meeting, Cancun, Mexico.

INVITED PODIUM PRESENTATIONS

1. **Goneau LW**, Hannan TJ, Hultgren SJ, Burton JP, Razvi H, Reid G. (2014) Time to re-evaluate antibiotic prophylaxis for recurrent urinary tract infection. London Health Research Day, London, Canada. Featured in the London Free Press Newspaper: http://www.lfpress.com/2014/03/18/study-by-western-phd-candidate-suggests-inappropriate-use-of-antibiotics-could-make-matters-worse-in-some-infections.

2. **Goneau LW**, Cadieux PA, Burton J, Reid G. (2012) Persister cell traits of uropathogenic bacteria in sepsis. The Infection and Immunity Research Forum Annual Meeting, The University of Western Ontario, London. Canada. Selected for Cedar Lane and Novus Biologicals 'Best in Show' Oral Presentation Award.

3. **Goneau LW**, MacDonald K, Razvi H, Cadieux PA. (2012) Sub-Minimal Inhibitory Concentrations of Antimicrobials Prime *Staphylococcus saprophyticus* for Survival Within the Urinary Tract During Prophylaxis. The Northeastern Section of the American Urology Association Annual Meeting, Niagara Falls, Canada.

4. **Goneau LW**, MacDonald KW, Razvi H, Cadieux P. (2012) Sub-Minimal Inhibitory Concentrations of Antimicrobials Prime *Staphylococcus saprophyticus* for Survival Within the Urinary Tract During Prophylaxis. The American Urology Association - Society for Infection and Inflammation in Urology Annual Meeting, Atlanta, USA.

5. **Goneau LW**, MacDonald KW, Cadieux PA. (2011) Antimicrobial Induced Tolerance Mechanisms in *Staphylococcus saprophyticus* and their Role in Recurrent and Device Associated Urinary Tract Infection. The Infection and Immunity Research Forum Annual Meeting, The University of Western Ontario, London. Canada.

INVITED PODIUM PRESENTATIONS (continued)

6. **Goneau LW**, Mendez-Probst C, Razvi H, Cadieux PA. (2010) Effects of Antimicrobials on Biofilm Formation in *Staphylococcus saprophyticus*. The World Congress of Endourology Annual Meeting, Chicago, USA.

INVITED SEMINAR PRESENTATIONS

1. **Goneau LW**, Cadieux PA. (2012) Hormetic Effects of Antibiotics on Bacteria and their Potential Role in Recurrent Urinary Tract Infection. Talks on Fridays (TOFs). LHRI, London, Canada.

2. **Goneau LW**, Cadieux PA. (2011) Sub-Inhibitory Antibiotic Treatment Induces High Density Growth and Improves *Staphylococcus saprophyticus* Survival Against Bactericidal Concentrations. Talks on Fridays (TOFs). LHRI, London, Canada.

3. **Goneau LW**, Cadieux PA. (2010) Effects of Stress on Biofilm Formation in *Staphylococcus saprophyticus*. Talks on Fridays (TOFs). LHRI, London, Canada.

POSTER PRESENTATIONS

1. **Goneau LW**, Yeoh N, Cadieux P, Burton JP, Razvi H, Reid G. (2013) Management of Recurrent Urinary Tract Infections: Something's Wrong with Diagnosis and Treatment. The Canadian Urological Association Annual Meeting, Niagara Falls, Canada.

2. **Goneau LW**, MacDonald K, Razvi H, Cadieux PA. (2012) Sub-Minimal Inhibitory Concentrations of Antimicrobials Prime *Staphylococcus saprophyticus* for Survival Within the Urinary Tract During Prophylaxis. The Northeastern Section of the American Urology Association Annual Meeting, Niagara Falls, Canada.

3. **Goneau LW**, Huynh MJ, MacDonald KM, Cadieux PA (2012) Effects of Sub-Minimal Inhibitory Concentrations of Antibiotics on Bacteria and their Potential Role in Recurrent Urinary Tract Infection. London Health Research Day, Infection and Immunity, London, Canada.

POSTER PRESENTATIONS (CONTINUED)

4. Huynh M, Bathini V, Fuller A, **Goneau LW**, MacDonald KW, Razvi H, Cadieux PA. (2012) Urinary Device Attachment of *Staphylococcus saprophyticus* in the Presence of Sub-Minimal Inhibitory Antimicrobial Concentrations. Canadian National Medical Student Research Symposium (CNMSRS), University of Manitoba, Winnipeg, Canada. Selected as Best in Show Presentation Award (New Trainee Category) (Co-author and supervisor).

5. Fuller A, **Goneau LW**, MacDonald KW, Erdeljan P, Bathini V, Razvi H, Cadieux PA (2011) Sub-Inhibitory Antibiotic Concentrations Enhance Surface Attachment, Survival and Host Immune Evasion in the Uropathogen *Staphylococcus saprophyticus*. Northeastern Section of the American Urological Association Annual Meeting, New Orleans, USA (Co-author).

6. **Goneau LW**, MacDonald KW, Erdeljan P, Fuller A, Fernandez A, Razvi H, Cadieux PA. (2011) Sub-Inhibitory Antibiotic Concentrations Enhance Surface Attachment and Survival in *Staphylococcus saprophyticus*. The Society for Infection and Inflammation in Urology Division of the American Urological Association Annual Meeting, Washington D.C., USA.

7. MacDonald KW, **Goneau LW**, Erdeljan P, Fuller A, Carreno A, Razvi H, Cadieux PA. (2011) Sub-Inhibitory Levels of Ciprofloxacin Enhance *Staphylococcus saprophyticus* Adherence to Bladder Cells and Reduce Pro-inflammatory Cytokine Expression. The Society for Infection and Inflammation in Urology Division of the American Urological Association Annual Meeting, Washington D.C., USA (Co-author).

8. **Goneau LW**, MacDonald KW, Cadieux PA. (2011) Sub-Inhibitory Antibiotic Treatment Induces High Density Growth and Improves *Staphylococcus saprophyticus* Survival Against Bactericidal Concentrations. The Annual Infection and Immunity Research Forum, Department of Microbiology and Immunology, Schulich School of Medicine and Dentistry, University of Western Ontario.

9. **Goneau LW**, MacDonald KW, Cadieux PA. (2011) Antimicrobial Induced Tolerance Mechanisms in *Staphylococcus saprophyticus* and their Role in Recurrent and Device Associated Urinary Tract Infection. Lawson Research Day. Clinical Investigations and Therapeutics, London, Canada.

10. **Goneau LW**, Mendez-Probst C, Razvi H, Cadieux PA. (2010) Effects of Antimicrobials on Biofilm Formation in *Staphylococcus saprophyticus*. The World Congress of Endourology Annual Meeting, Chicago, USA.

POSTER PRESENTATIONS (CONTINUED)

11. **Goneau LW**, Cadieux PA. (2010) The Effect of Stress on *Staphylococcus saprophyticus* Surface Attachment and Subsequent Biofilm Formation. Lawson Research Day. Clinical Investigations and Therapeutics, London, Canada.

12. **Goneau LW**, Wignall GR, Vanjecek M, Cadieux PA. (2009) Investigating biofilm formation, structure and antibiotic resistance in uropathogens. The Annual Infection and Immunity Research Forum, Department of Microbiology and Immunology, Schulich School of Medicine and Dentistry, University of Western Ontario.