Molecular Mechanisms of Group B *Streptococcus* Urinary Tract Infection and Adaptability to Growth in Human Urine

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Abstract

Bacteriuria, or the presence of bacteria in urine, is associated with both asymptomatic, as well as symptomatic urinary tract infection (UTI) and underpins much of the dynamic of microbial colonization of the urinary tract. The prevalence of bacteriuria in dissimilar patient groups such as healthy adults, institutionalized elderly, pregnant women, and immune-compromised patients varies widely. In addition, assessing the importance of 'significant bacteriuria' in infected individuals represents a diagnostic challenge, partly due to various causal microbes, and requires careful consideration of the distinct etiologies of bacteriuria in different populations and circumstances. Recent molecular discoveries have revealed how some bacterial traits can enable organisms to grow in human urine, which, as a fitness adaptation, is likely to influence the progression of bacteriuria in some individuals.

This study was designed as a comprehensive analysis of asymptomatic bacteriuria (ABU) causal organisms in dissimilar populations, and an in-depth microbiological analysis of the mechanisms used by one such causal organism, *Streptococcus agalactiae*. This organism causes UTI including ABU; however, growth of *S. agalactiae* in human urine has not been reported. In the first part of this study, we evaluate the prevalence and etiology of bacteriuria, and discuss recent advances in the molecular detection of bacteriuria from a diagnostic viewpoint. The second part of this study was experimental, and includes several investigational approaches that analysed different *S. agalactiae* isolates from different patient groups, and assessed how this causal organism initiates ABU and UTI at the molecular level. A large part of this experimental work was designed to provide an insight into the diverse human urine growth phenotypes of many *S. agalactiae* isolates with different clinical history and propose a standardized synthetic human urine (SHU) that was used experimentally to study in-detail about this growth phenotype in a more controlled manner.
S. agalactiae bacteriuria is usually asymptomatic with either high (>10^4 CFU/ml) or low (<10^4 CFU/ml) counts in urine. In this work we screened three-hundred and fifty-eight S. agalactiae isolates from patients with either low-grade or high-grade ABU, or acute UTI to analyse their ability to grow in human urine. The results demonstrated that certain S. agalactiae isolates are highly adapted for fast growth in human urine whereas other isolates are unable to survive in urine. For example, we identified a novel phenotype of robust growth in human urine by ABU S. agalactiae (ABSA) that was not observed among uropathogenic S. agalactiae (UPSA). These data suggest that phenotypic diversity among S. agalactiae isolates for urine growth may contribute to different clinical conditions of UTI and especially ABU. In addition, other urine growth assays also emphasised the importance of performing both colony counts and turbidity measurements simultaneously to analyse the growth of streptococcus in urine.

Recent in vitro molecular discoveries by other researchers have elucidated how key bacterial traits can enable organisms to survive and grow in human urine as a means of microbial fitness adaptation for UTI. Several microbial characteristics that confer bacteruric potential have been identified in past studies including de novo synthesis of guanine, and relative resistance to D-serine. This study describes detailed molecular and metabolic analysis of selected S. agalactiae isolates that were shown to exhibit robust urine growth. This included genome sequencing, metabolic profiling and serotyping. In our study, comparative phenotypic profiling of ABSA 1014 (an ABU strain) and UPSA 807 (a uropathogenic strain isolated from a patient with cystitis) using phenotype metabolic arrays of >2500 substrates and physiologic conditions revealed unique and specific L-Malic acid catabolism in ABSA 1014. Subsequent whole-genome sequencing revealed divergence in malic enzyme-encoding genes between ABSA 1014 and UPSA 807 predicted to functionally impact the malate metabolic pathway in multiple UPSA strains. Furthermore, urine growth experiments comparing wild-type ABSA and a malic enzyme (maeE)-deficient mutant, which was functionally inactivated
for the malate metabolic pathway, revealed significantly attenuated growth of the mutant in normal human urine (NHU) and SHU containing malic acid. This shows that *S. agalactiae* growth in human urine relates, at least in part, to malic acid metabolism.

This thesis also compared the ability of ABSA and UPSA to establish urine and bladder colonization in the frequently used UTI C57BL/6 mouse model, and investigated the influence of bacterial malic acid metabolism on mouse bladder colonization. These results demonstrated that wild type ABSA can establish better bladder colonization than wild type UPSA or mutant ABSA strain deficient in the malate oxidoreductase encoding gene, *maeE*. Using a 23-target multiplex protein assay the study also showed elevated production of cytokines and chemokines in the mouse bladder in response to ABSA and UPSA infections. Four main patterns of cytokine production were observed with significant differences between ABSA and UPSA such as for IL-10 and IL-17A.

Finally, pilot experiments were conducted to analyse the role of iron acquisition in *S. agalactiae* growth in human urine. This study showed that growth of ABSA 834 in human urine is independent of iron availability in human urine. Screening of virulence genes related to iron including *fhuD* and other genes involved in metabolism such as *guaA* showed the presence of multiple virulence-associated genes in ABSA 834 and reference *S. agalactiae* strains. Experiments also revealed a role for the gene *guaA* (encodes guanosine monophosphate) in supporting the growth of ABSA in human urine by generating and testing a ABSA 834 *guaA* mutant in growth assays. Collectively, the work described in this thesis provides key new insights into the molecular mechanisms of *S. agalactiae* UTI and, especially, the mechanisms underlying ABU caused by this important human pathogen.
Statement of originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

(Signed)_______________________________

Deepak Samuel Ipe
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABSA</td>
<td>Asymptomatic Bacteriuria <em>Streptococcus agalactiae</em></td>
</tr>
<tr>
<td>ABU</td>
<td>Asymptomatic Bacteriuria</td>
</tr>
<tr>
<td>ACP</td>
<td>Alpha C Protein</td>
</tr>
<tr>
<td>ACT</td>
<td>Artemis Comparison Tool</td>
</tr>
<tr>
<td>B-h/c</td>
<td>Beta hemolysin/Cytoxin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>CAMP</td>
<td>Christine Atkins Munch Peterson</td>
</tr>
<tr>
<td>CC</td>
<td>Clonal Complexes</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>Cm</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyl Trimethylammonium Bromide</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide Triphosphates</td>
</tr>
<tr>
<td>Erm</td>
<td>Erythromycin</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>Fhu</td>
<td>Ferric Hydroxamate</td>
</tr>
<tr>
<td>Fsba</td>
<td>Fibrinogen Binding Protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GAS</td>
<td>Group A Streptococcus</td>
</tr>
<tr>
<td>GBS</td>
<td>Group B Streptococcus</td>
</tr>
<tr>
<td>GI</td>
<td>Gastro-intestinal</td>
</tr>
<tr>
<td>IL-1α</td>
<td>Interleukin-1 alpha</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid Chromatography Mass Spectrometry</td>
</tr>
<tr>
<td>LMP</td>
<td>Laminin Binding Protein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>Macrophage Inflammatory Protein-1 beta</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Manganese</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>OAB</td>
<td>Overactive Bladder</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PM</td>
<td>Phenotype Metabolic</td>
</tr>
<tr>
<td>PMNs</td>
<td>Polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>SHU</td>
<td>Synthetic Human Urine</td>
</tr>
<tr>
<td>SodA</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>Spt</td>
<td>Spectinomycin</td>
</tr>
<tr>
<td>Srr</td>
<td>Serine Rich Repeats</td>
</tr>
<tr>
<td>ST</td>
<td>Sequence Type</td>
</tr>
<tr>
<td>sUTI</td>
<td>Symptomatic Urinary Tract Infection</td>
</tr>
<tr>
<td>Tet</td>
<td>Tetracyclin</td>
</tr>
<tr>
<td>THB</td>
<td>Todd-Hewitt Broth</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris-Hydrochloride</td>
</tr>
<tr>
<td>THP</td>
<td>Tamm-Horsfall protein</td>
</tr>
<tr>
<td>UPEC</td>
<td>Uropathogenic <em>E. coli</em></td>
</tr>
<tr>
<td>UPSA</td>
<td>Uropathogenic <em>Streptococcus agalactiae</em></td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary Tract Infection</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
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</table>
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Acknowledgement of published papers included in this thesis

Section 9.1 of the Griffith University Code for the Responsible Conduct of Research (“Criteria for Authorship”), in accordance with Section 5 of the Australian Code for the Responsible Conduct of Research, states:

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- Conception and design of the research project
- Analysis and interpretation of research data
- Drafting or making significant parts of the creative or scholarly work or critically revising it so as to contribute significantly to the final output

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- Include in the list of authors only those who have accepted authorship
- Appoint one author to be the executive author to record authorship and manage correspondence about the work with the publisher and other interested parties.
- Acknowledge all those who have contributed to the research, facilities or materials but who do not qualify as authors, such as research assistants, technical staff, and advisors on cultural or community knowledge. Obtain written consent to name individuals.
Included in this thesis are papers in Chapters 2 and 4 which are co-authored with other researchers. My contribution to each co-authored paper is outlined at the front of the relevant chapter. The bibliographic details / status for these papers including all authors, are:

Appearing in Chapter 2:


   Supplementary Data in Appendix 2.

Appearing in Chapter 4:


Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in each published paper.

(Signed) _________________________________
Deepak Samuel Ipe

(Countersigned) ___________________________
Supervisor: Associate Professor Glen Charles Ulett
Additional publications being prepared using work described in this thesis are:


**Ipe DS, Sullivan MJ, Ben Zakour N, Beatson SA, Davies MR, Dougan G, Ulett GC.** Asymptomatic bacteriuria due to *Streptococcus agalactiae* is associated with bacterial fitness for growth in human urine independent of iron acquisition. FEMS Microbiology Letters (in preparation)

**Ipe DS, Ben Zakour N, Beatson SA, Ulett KB, Benjamin WH Jr, Davies MR, Dougan G, Ulett GC.** Asymptomatic bacteriuria *Streptococcus agalactiae* demonstrate superior growth fitness for human urine across diverse serotypes and sequence types. Journal of Infectious Diseases (in preparation)
Chapter 1

General Introduction

The genus Streptococcus includes numerous pathogenic and non-pathogenic species. β-hemolytic streptococci, termed group A and group B *streptococci* for their respective Lancefield antigens (1, 2), readily lyse red blood cells and cause various fulminating infections in humans. Organisms that express the group B Lancefield antigen, identified as *S. agalactiae* or group B *streptococcus* (GBS), are common inhabitants of the gastro-intestinal (GI) and female urogenital tract. This organism was first identified as a cause of bovine mastitis however an epidemiology study in 1977 (3) revealed that *S. agalactiae* was also associated with human disease, in particular, postpartum infection in new-borns. *S. agalactiae* asymptomatically colonizes the GI and female genitourinary tracts in 25-50% of healthy adults (4, 5). Approximately 15%-35% of pregnant women harbor *S. agalactiae* in the GI and/or genitourinary tract during pregnancy (6). Colonization can occur as intermittent, transient or persistent infection, which can last for months (6-8). Neonatal disease due to *S. agalactiae* including pneumonia, meningitis, and sepsis stems from vertical transmission of the bacterium from the urogenital tract of colonized mothers to their infants (9). Transmission of *S. agalactiae* to neonates from their asymptomatically colonized mothers occurs in approximately 1% of all live births (6, 10, 11). Disease in adults often presents as pneumonia, sepsis or skin and soft tissue infections, which have increased in incidence over the past twenty years (12). The number of reports on the incidence of genitourinary infections due to *S. agalactiae* have increased over the past decade and UTIs have increasingly been associated with infection in the elderly, immunocompromised and adults with chronic medical illness (11-16).
Epidemiology and clinical manifestations

The changing spectrum of disease due to *S. agalactiae* in adults over the past decade has been highlighted by several prior studies (11, 13, 17, 18). Over 60% of cases of invasive *S. agalactiae* disease in the United States now occur in adults and most are unrelated to pregnancy (19). Thus, *S. agalactiae* is now less frequently linked to puerperal infections and more commonly associated with disease in non-pregnant adults. The most common clinical manifestations in adults are skin and soft tissue infections, bacteremia, pneumonia, arthritis, endocarditis and UTI (20-23). Thus, the quasi-commensal nature of *S. agalactiae* in the urogenital tract of symptomatically colonized women starkly contrasts with the acute virulence-related infections that *S. agalactiae* causes in newborns and with increasing incidence, adults (24, 25). Disease is especially common among adults with diabetes, malignancies and the elderly and immunocompromised (12, 26). UTI due to *S. agalactiae* is especially prevalent among nursing-home residents (22, 26-28). The overall case fatality rate for *S. agalactiae* infection in elderly adults was estimated at 15% in the United States between 2001-2005 (12, 26). Recurrent infections occur in approximately 5% of non-pregnant adults who develop a second episode of disease after resolution of primary infection (13). Some of the most common co-morbidities include diabetes mellitus, malignancy (29), cardiovascular abnormalities, genitourinary disorders, neurologic deficits, cirrhosis, steroid use, AIDS, renal dysfunction and peripheral vascular disease. There is no effective immunization strategy or vaccine against *S. agalactiae* (30, 31).

The spectrum of UTIs caused by *S. agalactiae* include ABU, cystitis, pyelonephritis, urethritis, and urosepsis (12, 16, 32-36). An unusual acute presentation related to a massive abdominopelvic abscess was recently reported (37). In most cases, colonization of the urinary tract probably occurs by an ascending route from the genital tract (16). Generally, *S. agalactiae* is cultured from 1.5-2.5% of all clinically suspected cases of UTI (34, 38, 39); however, some studies have reported isolation rates ranging...
between 5.7%-39.4% in distinct patient populations (22, 40). The highest isolation rates have related mostly to elderly populations and nursing home residents (12, 14, 17, 18, 22). Other studies have reported that UTI may account for up to one-third of all invasive infections due to *S. agalactiae* in elderly adults (15, 17, 34, 41). In a single-center analysis of adults in the United States, *S. agalactiae* was cultured from urine during routine UTI assessment in 2% of all cases (16), which is a prevalence rate that aligns closely with another prior study (42). Follow up over a 4-year period confirmed that most of these individuals represented ABU (43). Those most at risk for the development of acute UTI due to *S. agalactiae* are middle-aged to elderly individuals (12, 17, 18). Increased age was significantly associated with acute infection in a recent study, which reported the mean age of acutely infected adults as 53 years (range 19-93 years) (16). Risk factor associations have linked *S. agalactiae* UTI with diabetes in two studies (37, 44) but diabetes was not a risk factor in the most recent and largest study cohort (16). Neurogenic bladder (45), urinary tract abnormalities, chronic renal failure (34), corticosteroid use (17) and prior history of UTI (16) are other identified risk factors. Finally, UTI complicated 7% of cases of late-onset disease in neonates in one study (19).

**S. agalactiae** UTI during pregnancy

Up to 7% of pregnancies are reported to be complicated by *S. agalactiae* UTI, which also accounts for approximately 10% of all cases of pyelonephritis during pregnancy (18, 46, 47). In relation to pregnancy *S. agalactiae* UTI may contribute to chorioamnionitis (48), premature onset of labour (49), and increased risk of vertical transmission to newborns (50, 51). *S. agalactiae* ABU is of particular interest because this has been associated with vertical transmission and an increased risk for early-onset disease in newborns (20, 46, 49-52). One study found an elevated risk for early-onset disease among infants born to women with low colony-count *S. agalactiae* ABU compared to mothers who did not have *S. agalactiae* ABU (53). The prevalence of *S.
agalactiae ABU (including pure and predominant growth of S. agalactiae in the urine) during pregnancy remains unclear. Isolation rates from urine cultures of pregnant women have been reported at between 1%-3.5% in several studies (27, 36, 54). In one study, the rate of pregnancy among women with S. agalactiae-positive urine cultures was 25-30% (43). Others that have reported isolation rates between 2%-7% have considered positive cultures from urine as a surrogate marker for heavy genital tract colonization (20, 36, 46, 49, 51) and an indicator for intrapartum antibiotic prophylaxis (36, 55).

Most data on the risk for early-onset disease among infants born to mothers with S. agalactiae ABU are derived from studies using thresholds >10⁵ CFU/ml (46, 50, 51). However, low counts of S. agalactiae (10³-10⁴ CFU/ml) in urine have been associated with acute and persistent UTI (16), and colonization (56). The most effective approach for screening pregnant women for S. agalactiae ABU remains undefined. ABU during pregnancy has been indicated for intrapartum antibiotic prophylaxis since 1996 (55, 57) because it is considered a risk factor for neonatal disease and preterm labor (55, 57). Several guidelines recommend screening of pregnant women in the third trimester and antibiotic therapy in cases of positive urine cultures for S. agalactiae ABU (58-60). The most recent guidelines recommend reporting positive cultures at any count in urine (61). This approach represent an increased workload for clinical laboratories that generally do not report bacterial growth in urine of other pathogens at concentrations <10⁴ CFU/ml (62). In addition, most clinical laboratories rarely know whether urine samples are from pregnant women. In the context of universal late antenatal S. agalactiae screening, it is unclear how much early-onset disease is prevented by screening for low colony-count ABU as per the current recommendations, and whether reporting of such bacteriuria is cost-effective. In addition, studies have shown that women who exhibit positive S. agalactiae urine cultures during the first trimester of pregnancy may not exhibit vaginal-rectal colonization at 35-37 weeks gestation (36), or
at the time of delivery (63). Finally, although pregnant women may receive antibiotics to treat *S. agalactiae* ABU this may not eliminate *S. agalactiae* from the genitourinary tract. This has been shown in several studies that have reported recolonization following antibiotic therapy (64-66). Thus, current data indicates the link between low colony-count ABU during pregnancy and an increased risk for early-onset disease in infants is unclear and requires further study (46).

**Serotypes associated with UTI**

There are ten different capsular serotypes of *S. agalactiae*: Ia, Ib, and II-IX. Capsular serotyping can be performed by latex agglutination (67) or molecular serotyping (68-72) that uses a multiplex PCR and reverse line blot hybridization assay to target the species-specific *cfb* gene and serotype-specific sequences in capsular loci (73). Serotypes Ia, II, III and V are most commonly associated with *S. agalactiae* invasive disease (12, 16). In a study of non-pregnant adults Skoff *et al.*, showed that serotypes Ia, II, III and V accounted for 78.5% of almost two-thousand infections in the period between 2005-2006 (74). Serotype V is estimated to account for approximately one third of clinical isolates in the United States (75, 76). In a 2005 study of colonization of healthy elderly adults the most prevalent serotypes were: V (47.3%), Ia (22.8%), III (12.3%) and nontypeable *S. agalactiae* (12.3%) with an overall colonization rate of 21.7% (77). There have been a few studies on the serotypes associated with UTI and these indicate substantial diversity in the range of serotypes that cause acute infection. Generally, studies have shown that serotypes Ia, II, III, and V (16) cause most acute UTI (36, 50). The largest study, based on 387 patients in the United States reported the most prevalent serotypes associated with UTI according to molecular serotyping were Ia, III and V. These three serotypes accounted for 76% of isolates associated with UTI. Serotype III was responsible for a disproportionately high number of acute UTI cases in that study and was the only serotype with this disease association (16). There have been notable differences in the isolation rates reported for nontypeable strains from
cases of UTI. For example, nontypeable strains accounted for up to 30% of isolates in two UTI studies (36, 50) but there were no nontypeable strains associated with UTI in a more recent, larger cohort study that used molecular serotyping (16). These findings may reflect true differences in serotype distributions between studies but also point to the limitations of agglutination serotyping approaches for some in S. agalactiae epidemiological surveys as previously discussed (16). Switching between capsular serotypes may also occur in S. agalactiae (78) but this has not been investigated in the context of UTI.

**Virulence factors**

A number of S. agalactiae virulence factors have been characterized in detail but only capsule and the β-hemolysin/cytolysin (β-h/c) have been examined in the context of UTI pathogenesis. Several other exotoxigenic virulence factors are also produced by S. agalactiae, including hyaluronate lyase, Christine Atkins Munch Peterson (CAMP) factor, superoxide dismutase, proteases, nucleases, platelet-activating factor, collagenase/oligopeptidase, protein c, RIB, R protein and C5a peptidase. The roles of these factors in S. agalactiae virulence are reviewed elsewhere (79-81).

**Adherence of S. agalactiae to host cells**

Several of the aforementioned virulence factors are involved in recognition of S. agalactiae by host cells and/or inducing or evading immune responses (80, 82-84) and are reviewed elsewhere (80). As with most S. agalactiae infections UTI involves binding of the bacteria to epithelial cells at the mucosal surface as an initial step in pathogenesis. In the genital tract, S. agalactiae binds to human vaginal epithelial cells under low pH conditions, which involves low avidity interactions of LTA and high affinity interactions of hydrophobic S. agalactiae surface proteins (85, 86). The interactions that mediate S. agalactiae adherence to bladder mucosa have yet to be defined. UPSA have been shown to bind to both murine and human bladder uroepithelium in vivo and
in vitro, and UPSA was shown to bind more efficiently to uroepithelial mucosa than non-uropathogenic S. agalactiae (87). More recently, the binding of UPSA was compared to UPEC in experimental models using human bladder cells and murine tissue and found to be similar (88). S. agalactiae adhesion to extracellular matrix proteins such as fibronectin, fibrinogen and laminin can aid binding to host-cell-surface proteins such as integrins (89) but the role of these proteins in UTI have not been examined. S. agalactiae ScpB, a surface protein characterized for its ability to cleave C5a (90), can bind fibronectin (83) and integrins that probably promotes binding to host cells and complement proteolysis (91). Naturally occurring enzymatically-inactive ScpB variants retain the capacity to bind fibronectin separating the adhesive and proteolytic activities of ScpB (92, 93). The surface-anchored protein FbsA mediates attachment of S. agalactiae to fibrinogen (94), whereas binding to laminin involves the adhesin Lmb (95). The serine-rich repeat domain protein Srr-1 binds human keratin 4 (96), whereas the S. agalactiae surface protein LrrG, containing the leucine-rich-repeat motif found in many invasins, binds to epithelial cells (97). The possible role of these adhesins (i.e. ScpB, FbsA, Lmb, Srr-1, LrrG), and more recently described surface factors (e.g. pili, BasB, GAPDH) (98, 99) in binding to uroepithelial cells is unknown.

**Capsule**

Capsular polysaccharides present on the surface of S. agalactiae are major virulence factors involved in pathogenicity and survival in the host (100). Capsule layers form thick barriers that are essential for defense. Variations in four carbohydrate moieties (glucose, galactose, N-acetyl-glucosamine and N-acetyl-neuraminic acid) give rise to the ten S. agalactiae serotypes (12). The biochemical synthesis of capsule is a complex process encoded by a multitude of cps and neu genes, and variations in enzyme activities and operon structure can lead to variable encapsulation and phagocytosis resistance. Although capsule structures vary widely in composition and length, sialic acid residues at the termini of capsular polymers are ubiquitous (101).
These sialic acid residues are structurally identical to host cell surface glycoproteins and are well characterized for their role in a variety of mechanisms contributing to the evasion of host immunity (102-106). These residues are recognized by members of the Sialic acid Immunoglobulin-like lectin (Siglecs) superfamily (107), present on the surface of leukocytes. Interactions between sialic acid and Siglecs can subvert pathogen recognition responses and dampen inflammatory responses such as the oxidative burst (102, 103, 108). Mammalian host cells can utilize sialidases to remove sialic residues from the capsule of bacteria as a defense mechanism, but remarkably, *S. agalactiae* can counteract this by further structural modification. By O-acetylation of capsule sialic acid using O-acetyltransferase/sialic acid synthetases, *S. agalactiae* can modify the capsule to selectively mimic host cell glycoproteins (103, 109, 110). This represents a trade-off between displaying sialic acid for leukocyte manipulation, and O-acetylation these residues to prevent their removal by sialidases (and avoid leukocyte-killing), without compromising (capsule-driven) evasion from complement. Thus, *S. agalactiae* capsule provides a virulence mechanism (resistance to leukocyte killing) and an immune evasion strategy (resistance to complement).

In a recent study by Kline et al., using a murine model to study UTI, *S. agalactiae* was shown to preferentially colonize the kidney and induce inflammation, leukocyte recruitment and persistence for up to 14 days. In contrast, *S. agalactiae* colonization of the bladder diminished after 24 h post infection in the absence of notable inflammation or leukocyte responses. These data suggest that *S. agalactiae* can suppress the inflammatory response during cystitis (103). Elevated levels of pro-inflammatory cytokines including interleukin-1α (IL-1α), IL-9, macrophage inflammatory protein-1α (MIP-1α), and MIP-1β were detected in bladder, as was the master regulatory cytokine IL-10 (111). Detection of high concentrations of IL-1α and IL-10 in bladders of *S. agalactiae*-challenged mice is consistent with several previous studies (87)(and
unpublished; Chapter 6). When polymorphonuclear leukocytes (PMNs) were challenged with S. agalactiae deficient in neuA (a mutation that leads to hyper O-acetylation of capsule sialic acid residues) the PMNs-produced IL-1α, TNF-α, IL-1β, KC and MIP-1α at significantly higher levels in response to neuA-deficient S. agalactiae. Other studies have shown that capsular polysaccharides strongly induce the production of pro-inflammatory cytokines including TNF, IL-1 and IL-6 (112, 113). Thus, these data imply a role for capsule sialic acid residues in the suppression of pro-inflammatory cytokines (103) and a key role of S. agalactiae capsule in driving inflammation during UTI.

β-hemolysin/cytolysin (β-h/c)

The hemolysins of Streptococcus are potent exotoxins that contribute to virulence. Of the two S. agalactiae hemolysins, CAMP and β-h/c, only β-h/c, which is surface located (114) and expressed by almost all clinical isolates in varying amounts (81, 115) has been studied in UTI pathogenesis. The β-h/c was first defined as a pore-forming exotoxin responsible for the zone of clearing surrounding colonies on blood agar (116). The cyl locus was identified as being responsible for conferring the β-hemolytic activity to S. agalactiae (95, 117). Initially, the product of cylE was reported to be the toxin (117). More recent evidence has shed light onto the mechanisms underlying the expression of this elusive exotoxin and has been reviewed elsewhere (118). Functional cylE is linked to the co-expression of a carotenoid-like compound, granadaene, which confers pigmentation to colonies grown on agar (115, 116). Carotenoid pigment increases S. agalactiae resistance against host defenses including oxidative damage, while cylE-deficiency increases susceptibility to killing by blood, macrophages and neutrophils (115). However, recent data that cylE-deficient S. agalactiae was equivalent to wild type (WT) bacteria in intracellular survival in macrophages (119) argues for a complex role of the β-h/c in different host niches. Structural (120) and
functional studies (121) of purified pigment showed that it is sufficient to lyse red blood cells indicating that $\beta$-h/c is granadaene and not a polypeptide product of the cylE locus.

In general, $\beta$-h/c is well documented for a role in inducing pro-inflammatory responses (122-124) and invading host cells of the lung, brain and liver (124-128). $\beta$-h/c-deficient S. agalactiae strains are attenuated for virulence in various animal models of sepsis, meningitis, arthritis, genital tract colonization and pneumonia (125, 128-131). Furthermore, hyper-hemolytic S. agalactiae strains exhibit increased virulence, induce apoptosis (122, 132, 133) and are more frequently associated with pre-term labor and patients with S. agalactiae-associated toxic shock syndrome or necrotizing fasciitis (13, 121). $\beta$-h/c triggers early but not late apoptosis (134, 135) that may contribute to disease by promoting immune-evasion (136). Whilst this occurs in UPEC UTI and is related to alpha-hemolysin expression (137) apoptosis has not been reported in UTI due to S. agalactiae. Indeed, the role of S. agalactiae $\beta$-h/c in UTI appears to be distinct from its well-documented roles in other disease models. In contrast to disease in lungs, the blood-brain barrier and liver, data suggest the $\beta$-h/c has no notable role in colonization and invasion in the urinary tract. In two recent studies different $\beta$-h/c-deficient S. agalactiae mutants were as equally as effective at colonizing the urinary tract compared to WT bacteria in mice (103, 138). This suggests that cylE contributes little directly in the way of S. agalactiae survival in UTI. Nevertheless, $\beta$-h/c-deficient S. agalactiae failed to elicit the same degree of pro-inflammatory response in bladder epithelial cells and murine urinary tract tissue, suggesting a role for $\beta$-h/c in localized inflammation that may directly affect S. agalactiae UTI pathogenesis (138).

**Surface proteins and pili**
Several *S. agalactiae* surface proteins are implicated in virulence, attachment and adherence. Surface proteins can be identified by the presence of motifs involved in anchoring to the cell surface, including those that contain the LP(X)TG anchoring sequence, or those with a lipobox. Examples of the former include the pili (PilABC/pil-2B), Serine-rich repeat proteins (Srr-1/Srr-2), bacterial surface adhesion of *S. agalactiae* (BsaB), *S. agalactiae* immunogenic bacterial adhesion (BibA), Fibrinogen binding protein (FsbB/A), and the alpha-like proteins (alp; α-C protein / Rib). We will discuss *S. agalactiae* pili due to the known binding functions of *S. agalactiae* pili towards multiple epithelial cell types (139, 140) and the major contribution of pili in mediating adherence of Gram-negative bacteria such as *E. coli* to uroepithelial cells during UTI (141, 142).

Multi-genomic analysis of various *S. agalactiae* strains revealed the presence of two islands encoding structural genes for pili (143-145), namely pilus island 1 (PI-1) and 2, the latter existing in two isoforms (PI-2a and PI-2b). Among eight sequenced *S. agalactiae* genomes only six contained both pilus-encoding loci (144). Pili in *S. agalactiae* comprise three proteins; PilB forms the major structural polymer and bona fide pilin; PilA contains a Von Willebrand adhesion domain for adhering to eukaryotic cells and is distributed along the PilB backbone; PilC is a small pilus-associated protein localized at the base of the pilus (146). Thus, *S. agalactiae* pilus genomic islands include the genes encoding PilB, the LP(X)TG-motif-containing protein that polymerises to form the pilus backbone, and accessory proteins PilA and PilC (146, 147). For correct anchoring and assembly, pili require the action of transpeptidase-like sortases (e.g. SrtA/B/C) which anchor LP(X)TG-motif proteins to the peptidoglycan wall. Sortases are often encoded by genes adjacent to pilABC and are indispensable for pili formation. Recently it was shown that *S. agalactiae* deficient in either sortases, or PilA/PilC were less efficient in adhering to alveolar or intestinal epithelial cells in vitro, and the authors suggested that the Von Willebrand adhesion domain of PilA
mediates direct contact with human cells but is not required for biofilm formation (140). Epithelial cell adhesion is reduced S. agalactiae mutants lacking PilA or PilC, but not those lacking PilB (146). In this context, the main polymer, PilB, is not required for adhesion to eukaryotic cells, suggesting a paradigm whereby pili display adhesive properties but the bona fide pilin polymer is dispensible for cell adhesion. More recently, PilA was shown to bind collagen and promote interactions with α2β1 integrin of endothelial cells in the blood brain barrier (148). In addition, PilA-binding promotes chemokine responses including increased expression of IL-8, leading to increased neutrophil recruitment and inflammation. This has been related to the increased permeability of the blood brain barrier to S. agalactiae during the onset of meningitis (148). The role of S. agalactiae pili in binding in the urogenital tract is unknown.

**Laboratory diagnosis and identification**

Clinically, UTI due to S. agalactiae is indistinguishable from UTI caused by other uropathogens (18). The majority of S. agalactiae infections can be diagnosed through routine laboratory testing of clinical samples such as blood, cerebrospinal fluid, or aspirates from sites of local suppuration however UTI present a diagnostic challenge due to the high rates of genitourinary colonization in women and frequent contamination of urine specimens used for culture. Thus, clinical diagnosis is imperative for S. agalactiae UTI. Pure cultures that are obtained from urine specimens are rapidly identified by typical colony morphology on agar medium such as tryptic soy agar-5% sheep blood, and are tested for catalase that streptococci do not express. Isolates are grouped into the Lancefield B group (2) using commercial typing antisera for latex agglutination. S. agalactiae antigens can occasionally be detected in urine but are not routinely analyzed in diagnostic assays. Polymerase chain reaction or immunoassay may provide rapid and specific detection of S. agalactiae; however, validation of these assays for sensitivity and specificity has not been reported and thus, limited their widespread application in the clinical laboratory (149, 150).
Antibiotic treatment and resistance

In the 1980s the mortality rate from neonatal *S. agalactiae* sepsis was around 50% (151). Intrapartum antibiotic treatment reduced this incidence to 10%-15% (9, 19), and sustained implementation of treatment, awareness, screening and surveillance programs has continued to reduce mortality rates (58, 151, 152) that are now approximately 4-5% (12, 153-155). However, the success of these strategies depends on the approach to implementation (156, 157) and rates of *S. agalactiae*-related stillbirths, prematurity, and late onset disease have not decreased (158)(CDC, 2002). The incidence of morbidities in newborn survivors of acute infection also remains high (>20%) with frequent neurological sequelae (158, 159). Penicillin-derived antibiotics remain the drugs of choice in infants and adults (13, 153). Vancomycin, cefozolin, clindamycin and telavancin are also used. Success rates of antibiotic treatment strategies for *S. agalactiae* UTI have not been carefully examined; however, there are multiple studies showing that infections can be associated with relatively high rates of treatment failure and poor clinical outcomes (34). The emergence of *S. agalactiae* strains that are resistant to multiple antibiotics including penicillin, clindamycin and erythromycin (160-166), which may reflect clonal dissemination and horizontal transfer of resistance genes (167), is worrying (160-166, 168). These trends highlight the importance of *S. agalactiae* as a public health concern and the need for increased resistance monitoring.
The specific aims and hypothesis of this thesis are to:

1) Investigate the ability of *S. agalactiae* to grow in human urine and utilise human urine as an energy source that may be used by the bacterium to persist in the niche of host urinary tract or bladder and contribute to the pathogenesis of UTI (Chapter 4).

2) Identify potential differences between *S. agalactiae* isolates recovered from different clinical conditions for growth in human urine with a focus on Asymptomatic Bacteriuria *S. agalactiae* (ABSA) and Uropathogenic *S. agalactiae* (UPSA) (Chapter 4 and 5).

3) Identify potential correlations between the ability to grow in human urine and clinical presentation or disease severity in patients with UTI (Chapter 4 and 7).

4) Assess the utility and reliability of colony count and turbidity measures for assessing *S. agalactiae* growth in urine and determine the correlation between these two methods (Chapter 7).

5) Define the serotype distributions among *S. agalactiae* isolates that exhibit different growth phenotypes in human (Chapter 4).

6) Investigate the genetic basis for uropathogenicity in *S. agalactiae* by whole genome sequencing in a defined collection of UTI isolates (Chapter 5).

7) Determine the virulence phenotype and immunogenicity of representative *S. agalactiae* isolates from different patient groups for colonization in a mouse UTI model (Chapter 6).

8) Assess the contribution of selected target genes such as *maeE* and *guaA* of *S. agalactiae* in utilization of human urine (Chapter 5 and 7).
The hypotheses of this thesis are that:

1) Certain *S. agalactiae* can utilise human urine as a growth media and energy source and this may be a mechanism used by the bacterium to persist in the niche of the host urinary tract and contribute to the pathogenesis of UTI or ABU.

2) Phenotypic and genotypic differences exist between *S. agalactiae* isolates recovered from different patient groups (i.e. symptomatic versus asymptomatic UTI).

3) Particular genes contribute to the robust growth pattern of asymptomatic bacteriuria (ABU) *S. agalactiae* in human urine.

4) The colonization potential of ABSA and UPSA in mice is different and dissimilar *S. agalactiae* induce distinctive host immune responses in the bladder.
Chapter 2

Asymptomatic bacteriuria: prevalence rates of causal microbes, etiology of infection in different patient populations, and recent advances in molecular detection

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, is:


Supplementary material placed in Appendix 1.

My contribution to the paper involved:
The literature analyses, structure of the review and the manuscript writing

(Signed) _________________________________ (Date)______________

Deepak S. Ipe

(Countersigned) ___________________________ (Date)______________

Corresponding author of paper: Glen Charles Ulett

(Countersigned) ___________________________ (Date)______________

Supervisor: Glen Charles Ulett
Introduction

Bacteriuria in asymptomatic and symptomatic urinary tract infection

The term ‘Asymptomatic Bacteriuria’ (ABU or ASB) is effectively synonymous with asymptomatic Urinary Tract Infection (UTI) in defining the isolation of a specified semi-quantitative count of bacteria in an appropriately collected urine specimen from a person without signs or symptoms related to UTI (169, 170). The semi-quantitative count used for defining ‘significant bacteriuria’ is a matter of debate, and will be discussed in the section on diagnostics. Bacteriuria is not restricted to ABU; it is also used to characterize symptomatic UTI (sUTI) based on clinical presentation and semi-quantitative counts as a diagnostic marker for grading infection. In this review, we discuss bacteriuria related to ABU, and how it affects different patient populations. We review the diversity of causal microbes, and how distinct etiologies of bacteriuria occur in dissimilar populations. Finally, we summarize recent advances in molecular detection approaches for the diagnostic assessment of bacteriuria.

Establishment of ABU in an individual depends on the entry of an organism with bacteriuric potential into the urinary tract. Long-term ABU is defined as a persistent infection of greater than three weeks (to several years) of duration, and stems from an organism being sufficiently adapted to urine to survive host defenses. Bacteriuria can originate from the bladder or the kidneys. Differentiating bladder versus renal origin bacteriuria is challenging but important from an etiological viewpoint because of the high incidence of renal infection in some patient populations such as community-dwelling elderly adults (171). The potential for bacteriuria to be both an infection, and a risk factor for subsequent development of sUTI highlights the complex nature of the condition (172, 173). This helps to reconcile the contradictory roles of ABU in protecting some individuals against sUTI (174-176), but predisposing others to developing sUTI.
Clinically, treatment is not recommended as routine practice for ABU, excepting for pregnant women and individuals undergoing invasive genitourinary procedures. This is because of adverse events and a lack of efficacy in preventing subsequent sUTI (177). Instead, ABU is managed according to the clinical situation (178). A key concept is that a single definition does not sufficiently describe ABU from an etiological standpoint, which mirrors emerging concepts of imperfect definitions for complicated and uncomplicated sUTI (178). Hence, bacteriuria comprises an etiological spectrum that encompasses diverse causal bacteria with distinct prevalence rates in different patient groups, and involves different host factors that predispose to infection.

**Prevalence of ABU causal microbes in distinct patient populations**

Multiple species from fourteen genera cause essentially all culture-detectable ABU. Non-cultivable bacteria were recently described (179), and form part of the urine microbiome in healthy adults and those with neuropathic bladder (180, 181). Whether non-cultivable microbes influence bacteriuria due to cultivable organisms is unknown. *Escherichia coli* causes most ABU. Other Enterobacteriaceae (*Proteus mirabilis, Klebsiella pneumoniae, Enterobacter* spp., *Providencia stuartii, and Morganella morganii*), non-fermentative Gram-negative bacilli (e.g. *Pseudomonas aeruginosa*), and Gram-positive bacteria including *Enterococcus* spp., *Staphylococcus aureus*, and coagulase-negative staphylococci (mainly *Staphylococcus saprophyticus*) also cause ABU (44, 169, 182) (Table 2.1). ABU due to *Streptococcus agalactiae* is relatively common in pregnancy, particularly in the form of low count bacteriuria, and has been associated with adverse obstetric outcomes (48, 183, 184). This organism has also been a focus of several bacteriuria studies in non-pregnant adults (16, 41, 43). All of these species can also cause acute uncomplicated UTI (symptomatic bladder infection with frequency, urgency, dysuria, and/or suprapubic pain in a person with normal genitourinary tract function) (169, 178, 185).
The prevalence of ABU differs dramatically in distinct patient populations and is influenced by gender, age, medical interventions and comorbidities. Overall prevalence rates are summarized in Table 2.1, which illustrates how causal organisms disproportionately affect different patient populations. For example, *E. coli* is less prevalent among healthy men and patients with indwelling catheters; enterococci have been cultured from almost a quarter of bacteriuric healthy men, but only 3-4% of bacteriuric pregnant women. Staphylococci rarely cause ABU in healthy adults, but are relatively prevalent among ABU pregnant women, diabetic patients and community-dwelling elderly men. Detailed prevalence data are shown with supporting references in Supplementary Table S1. Why different bacteria disproportionately affect different patient populations is largely unknown. There are, however, unique frequencies of host characteristics in sUTI as defined by the causal organism (186) implying that host environment is important. Next, we will compare several etiologies of ABU: in pregnancy, diabetes, elderly patients, and controversies regarding women with urge incontinence.
Table 2.1: ABU prevalence in distinct patient populations (% below patient group), and comparative peak prevalence rates of causal bacteria

<table>
<thead>
<tr>
<th>Causal Organism</th>
<th>Healthy Adult Women (1-9%)</th>
<th>Healthy Adult Men (1-2%)</th>
<th>Pregnant Women (2-15%)</th>
<th>Diabetic Adults (1-30%)</th>
<th>Community Dwelling Elderly (2-50%)</th>
<th>Institutionalized Adults / Elderly (14-75%)</th>
<th>Patients with Indwelling Catheter (9-100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>91</td>
<td>25</td>
<td>86</td>
<td>80</td>
<td>80</td>
<td>60</td>
<td>35</td>
</tr>
<tr>
<td><em>Enterococcus spp.</em></td>
<td>33</td>
<td>23</td>
<td>4</td>
<td>13</td>
<td>8</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td><em>Staphylococcus spp.</em></td>
<td>2</td>
<td>7</td>
<td>24</td>
<td>16</td>
<td>53</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td><em>Proteus spp.</em></td>
<td>9</td>
<td>35</td>
<td>10</td>
<td>24</td>
<td>21</td>
<td>4</td>
<td>21</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>6</td>
<td>8</td>
<td>16</td>
<td>28</td>
<td>17</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>6</td>
<td>5</td>
<td>26</td>
<td>12</td>
<td>10</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><em>Pseudomonas spp.</em></td>
<td>12</td>
<td>6</td>
<td>18</td>
<td>14</td>
<td>14</td>
<td>25</td>
<td>21</td>
</tr>
<tr>
<td><em>Enterobacter spp.</em></td>
<td>3</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>21</td>
</tr>
<tr>
<td><em>Providencia spp.</em></td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>16</td>
<td></td>
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</tr>
<tr>
<td><em>Gardnerella vaginalis</em></td>
<td>15</td>
<td>4</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td><em>U. ureolyticum</em></td>
<td>15</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Morganella morganii</em></td>
<td>5</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td><em>Serratia spp.</em></td>
<td>5</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

1For causal organisms, peak prevalence is the highest reported prevalence rate from published studies; all % are rounded; references used are provided in...
Supplementary Table S1 that also presents % range, gender and age analyses where appropriate, alongside specific references; causal microbes grouped according to highest peak prevalence rate in at least one patient population [High (>30%), Moderate (>20%), Low (>10%) and Rare (≦10%)]. Shading key:

<table>
<thead>
<tr>
<th>&gt;80% of cases</th>
<th>30-80%</th>
<th>20-30%</th>
<th>10-20%</th>
<th>5-10%</th>
<th>2-5%</th>
<th>N/A</th>
</tr>
</thead>
</table>

N/A
Etiology of ABU in Pregnancy, Diabetes and Elderly Individuals

ABU in pregnancy relates to anatomical and physiologic changes in the urinary tract that alter the host environment. Compression of the ureters at the pelvic brim may predispose to upwards reflux of urine, thus ABU more readily progresses to pyelonephritis during pregnancy. Decreased concentration of urine, glucosuria and progesterone effects (promote ureteric dilatation) also influence infection (171). ABU prevalence rates in pregnancy range between 1.9-15% (Table S1). The risk of vertical transmission of bacteria is important, particularly given ABU is more persistent, and more frequently progresses to sUTI in pregnancy compared to other populations (e.g. 20-40% of untreated cases progress to sUTI, which is complicated by premature delivery in 20-50%) (171, 187). This may relate to lower antibacterial activity of urine during pregnancy (171). Routine culture-based screening at 12-16 weeks remains the consensus recommendation, and, despite evaluation of various screening tools (171, 188-190), the most effective approaches to screening and treatment continue to be reassessed (191, 192). E. coli causes up to 86% of cases, however, this varies widely; S. agalactiae is also important and was reported in 26% of cases in diabetes gravidas in one study (Table S1). S. agalactiae bacteriuria of any count in pregnancy necessitates antibiotic treatment. The benefits of treatment at low counts, however, remain uncertain (193). For other organisms, counts ≥10⁵ CFU/ml are treated according to the causal bacteria (184, 190, 193, 194). Screening and treatment reduces the risk of pyelonephritis and adverse obstetrical outcomes; however, the optimal duration of treatment is yet to be defined (61, 171, 190, 195).

The etiology of ABU in diabetes is interesting because ABU occurs three times more often in diabetic women compared to non-diabetic women, but no such association is found in men (196, 197). Risk factors include the duration of diabetes, degree of
metabolic control, diabetic complications including neuropathy and previous UTI (Table S1) (196, 198). *E. coli* is most frequently isolated, but Klebsiella and Proteus are also common. ABU in diabetes is difficult to eradicate, and up to 20% of untreated women remain bacteriuric with the original organism long-term (196). In women with type 2 diabetes, ABU is associated with a higher incidence of sUTI and urosepsis; however, studies have not shown any increased incidence of renal impairment long-term (172). While sUTI follows a more complicated course in diabetic patients, screening for ABU is not recommended because treatment does not reduce the incidence of sUTI or pyelonephritis (199). The impact of therapeuetic inhibitors of sodium-glucose transport proteins (that mediate 90% of glucose reabsorption in kidneys), such as canagliflozin, is unclear. Such drugs increase urinary glucose excretion, which was associated with increased sUTI in one study (200), however there was no association with increased ABU or sUTI in a randomized trial (201). Longer-term studies are needed to determine whether increased glucose availability (for microbes) in urine might contribute to progressive bacteriuria.

The etiology of ABU in elderly adults is multifactorial; risk factors include anatomic abnormalities (e.g. prostate obstruction), hormonal and metabolic changes (e.g. estrogen decrease, diabetes), neurologic disorders and poor peri-anal hygiene (202). ABU is especially prevalent among residents of long-term care facilities; up to 75% of institutionalized women, and 52% of men have been shown to be bacteriuric (Table S1). This contrasts to lower prevalence rates among community-dwelling elderly adults. Among non-*E. coli* causal organisms, *P. mirabilis*, Klebsiella, Pseudomonas and staphylococci are prevalent (Table S1). ABU in the elderly is not associated with either increased morbidity or mortality. Since treatment does not improve outcomes but frequently leads to reinfection with resistant organisms, neither screening nor treatment is recommended (169, 177, 203). In addition, distinguishing ABU from sUTI is difficult
because elderly patients have a smaller bladder capacity (thus often have frequency of micturition). Elderly patients also often have reduced fluid intake, which makes their urine concentrated and somewhat malodorous. Other atypical presentations are common, in that elderly patients with bacteriuria may simply appear unwell and confused; there is also a high prevalence of pyuria in asymptomatic individuals, which can complicate urinalysis interpretation (204).

**Current Controversy: Etiology of Bacteriuria in Women with Urge Incontinence**

The etiology of bacteriuria in women with urge incontinence (i.e. overactive bladder syndrome; OAB) is a current matter of debate. These individuals generally complain of frequency and urgency of micturition; suprapubic pain may be noted if the spasms of the detrusor muscle are intense. If such patients are found to have two cultures of the same species 24h apart, with bacteriuria $\geq 10^5$ CFU/ml of uropathogens (205), in the absence of dysuria, controversy now exists as to whether such women should be considered as having ABU, or in fact, a sUTI that warrants treatment. Recent evidence indicates that 26% of women with severe OAB may have $\geq 10^5$ CFU/ml without the classical features of dysuria or malodorous urine, compared to 4% of age-matched continent controls (206). The role of urine leakage onto the perineum, with possible facilitation of bacterial colonization of the urethral orifice remains controversial also, since patients with OAB leak urine intermittently, not continuously. Recent data regarding the treatment of bacteriuria in OAB showed significant benefit for urgency and frequency of micturition (207, 208) but a randomized controlled trial is currently pending.
**What factors influence the progression of bacteriuria?**

Recent reviews on the pathogenesis of sUTI (141, 209, 210) enable us to focus here on the progression of bacteriuria specifically. Whether short-term bacteriuria progresses to long-term ABU depends on both host and microbe traits. Overall, <1% of healthy adults with ABU will progress to long-term bacteriuria, and most will not harbor the same strain of causal organism over time (173). Thus, turnover of causal organisms is dynamic, and colonizing strains are often replaced by other strains during infection. For *E. coli*, long-term bacteriuria was recently shown to select for attenuated virulence phenotypes (211). Most microbes have low bacteriuric potential (i.e. cannot survive/grow in urine) and urine has natural antimicrobial properties, which probably impacts on strain replacement. The potential contribution of bacterial growth in urine to progression of ABU has been highlighted by several probiotic approaches aimed at reducing the incidence of sUTI (174-176, 212).

Host characteristics also influence the progression of bacteriuria. Clinically, for example, this is evidenced by the increased incidence of infection in asymptomatic elderly adults, which is disproportionally associated with residents of long-term care facilities (204). In part, these differences reflect the role of comorbidities such as neurological impairment in predisposing individuals to ABU (Table S1). Key differences in innate immune responses between individuals and genetic background are also important. For example, TLR4 promoter variants that are linked to reduced expression of TLR4 and reduced innate immune responses are associated with ABU, as recently reviewed (141). An evaluation of treatment data also offers some insight into the role of the host. In contrast to sUTI, which is resolved in most cases by treatment (213, 214), therapy is not routine for ABU (169, 177). While treatment can halt the progression of bacteriuria, and reduce the incidence of infection (215), patients infected with *E. coli*
experience re-colonization with the same or similar organism at surprisingly high rates (196). These data reveal the difficulty in achieving bacteriological cure for ABU, and imply that bacteriuria involves frequent re-colonization of the host. This is consistent with the view that ABU in adult women are seldom permanently eradicable (169). Thus, influences of both the microbe (survival/growth in urine, strain replacement) and host (comorbidities, urine anti-microbial activity, re-current infection, therapy) govern the progression of bacteriuria in infected individuals.

Advances in diagnostics: emerging molecular detection methods

Detection of bacteriuria in the diagnostic laboratory continues to rely on culture-based approaches, which alone have inadequate sensitivity and specificity to define ‘significant’ bacteriuria in diverse clinical scenarios (216). Indeed, there is no fixed number of significant bacteriuria that can be applied to all forms of UTI (including ABU) under all circumstances (205). The threshold of ‘significance’ for clean catch specimens was historically >10^5 CFU/ml, as reviewed elsewhere (170, 216); however, the importance of the clinical scenario has resulted in lower thresholds of >10^2 CFU/ml, >10^3 CFU/ml, and >10^4 CFU/ml for different conditions (178, 184, 216, 217). For example, the diagnostic cut-off for sUTI/ABU of 10^5 CFU/ml for patients with OAB has been lowered to 10^3 CFU/ml, as recommended by the European Association of Urology for Women (205). Lower thresholds improve sensitivity without undue impracticality, although have not yet translated widely into clinical practice (218). The importance of low counts for sUTI are discussed elsewhere (171, 219); but for ABU, the importance of low counts remains uncertain (171, 220). In healthy adult men, ABU is typically defined by a single voided specimen with one species ≥10^5 CFU/ml; in healthy adult women, two consecutive voided specimens with the same species ≥10^5 CFU/ml is used. In both groups, a single catheterized specimen with a species ≥10^2 CFU/ml
defines ABU (169). Problems of culture-based methods include low-count organisms coexisting with dominant organisms that tend to be missed by routine culture (221). Culture-based screening approaches also often fail to identify women who subsequently develop sUTI (171). Costs are also high given that urine samples are among the most numerous of specimen types sent for microbiology studies (US$2.20-2.45 per sample for agar/spot test reagents, excluding labor) (222). Other limitations of culture-based methods are accentuated in some patient populations. For example, atypical presentations in elderly adults who exhibit unusual symptoms of UTI (or none at all) makes interpretation difficult (223-225). Culture-based methods have been reported to miss up to half of ABU cases in pregnant women (226), and reports of “no growth” in symptomatic patients continue to be problematic (227).

Screening for prognostic markers that differentiate between ABU and sUTI supports the interpretation of culture results (228). Leukocyte esterase and pyuria as a marker of sUTI has been used for many years but is less useful in diabetic and elderly individuals. Dipstick analysis (for nitrites and esterase) and direct microscopy have poor positive and negative predictive values for ABU, and are of limited value in distinguishing sUTI (185, 229). Summaries of the sensitivities and specificities of some of these tests are reviewed elsewhere (184). Lactoferrin, proteinuria, microalbuminuria and volatile organic compounds have also been investigated to support culture results (230-232). Urinary interleukin-6 and -8 levels have shown some potential prognostic value for differentiating sUTI, especially in children, as reviewed elsewhere (233). Recently, a commercial system based on automated microscopy that analyzes cells and particles consistent with bacteria was shown to decrease the negative culture rate by >50%, but was reliant on operator input (234). Another recently reported flow cytometry-based system achieved similar sensitivity and specificity, and was able to reduce the number of urine cultures by 43% (235). These data compared with a high
rate of false negatives as reported by another group, which would preclude this method as a routine screening approach to exclude urine samples from culture (236). Overall, however, most flow cytometry-based studies reported so far have found few false positives and negatives, implying good potential for clinical application (237-240).

Aside from flow cytometry-based methods, new molecular detection methods that could reduce the need for urine culture and ease associated costs and workload are being developed. These are summarized in Fig 2.1. Several molecular detection methods are based on the detection of bacterial 16s rRNA with dsDNA probes, and molecular beacons with target sequences homologous to pan-prokaryotic rRNA or specific microbes. Using 16s rRNA is attractive because of relative target stability, and high copy number per bacterial cell. The use of fluorescent dsDNA probes achieved good sensitivity for low level bacteriuria in one study (241). However, like other molecular methods described to date, no clinical evaluation has been reported. Another promising method that was able to identify most uropathogens from urine is a system using 16s rRNA captured on gold film electrodes (242, 243).
Fig 2.1: Emerging Molecular Detection Methods for Bacteriruia
Fig 2.1: Emerging molecular detection methods for bacteriuria (A) Microparticle conjugated dsDNA probes for microbe-specific nucleic acid. When in the presence of the target a fluorophore probe is thermodynamically driven to hybridize to the target, which replaces a quencher probe. Probes are captured by streptavidin coated microparticles (241). (B) Electrochemical sensors illustrating (1) bacteria lysis to release 16S rRNA target (dashed line), (2) hybridization of target with fluorescein (green circle)-labeled detection probe (blue), (3) hybridization of target with biotin (red circle)-labeled capture probe (orange), (4) binding of anti-fluorescein antibody HRP-conjugate to target-probe sandwich, and (5) generation of current by transfer of electrons to the electron transfer mediator, TMB (242). (C) Molecular beacons to detect microbe-specific 16S rRNA. Similar to microparticle conjugates, these use spatial separation of fluorophore and quencher to enable a strong and sequence-specific increase in fluorescence in the presence of the target. Fluorescing molecular beacons hybridized to synthetic oligonucleotides are shown as a bright orange spot on a black background (244). (D) Quantum dot technology using cadmium detection, illustrating (1) sandwich complexes through dual hybridization, (B) dissolution of the assembled CdS-streptavidin quantum dots with nitric acid (HNO3), and (C) voltage monitoring of released Cd2+ (245). All panels reprinted with written permission: Riahi R, Mach KE, Mohan R, Liao JC & Wong PK, Analytical Chemistry 83: 6349-6354, Copyright (2011) American Chemical Society, Liao JC, Mastali M, Gau V, et al., Journal of Clinical Microbiology 44: 561-570, Copyright (2006) American Society for Microbiology, Bercovici M, Kaigala GV, Mach KE, Han CM, Liao JC & Santiago JG, Analytical Chemistry 83: 4110-4117, Copyright (2011) American Chemical Society, and Xiang Y, Zhang H, Jiang B, Chai Y & Yuan R, Analytical Chemistry 83: 4302-4306, Copyright (2011) American Chemical Society.
In this capture oligonucleotide method, universal bacterial 16s rRNA mixed with species- or group-specific labeled probes identified over 90% of uropathogens at very low levels within 40 min. Addition of lactoferrin detection to this system could enable the evaluation of pyuria. The labor and costs of such a system would be higher than culture, but would eventually be offset with automation and volume manufacturing (242, 243, 246, 247), as noted for microfluidics (248). Molecular beacons combined with 16s rRNA in a microfluidic chip in another study, using ‘isotachophoresis technology’, could analyze samples within only 15 min but were limited by a sensitivity of $10^6$ CFU/ml (244). For this approach, further improvements to sensitivity would likely enable coverage of other clinically-relevant bacteriuria ranges between $10^3$-$10^5$ CFU/ml.

An alternative molecular method to quantify bacteriuria without culture utilized quantum dot technology based on a cadmium ion electronic detection system (245). This system uses polystyrene beads covalently coated with Cd-streptavidin, which captures Cd-biotin-labeled poly-T-oligonucleotides. Biotin-labeled rRNA-specific capture probes are captured by the quantum dots that are captured by magnetic beads coated with a second 16s rRNA capture probe-specific for uropathogenic rRNA - when the magnetic bead and the quantum bead bind the same rRNA they are analyzed for Cd (Fig 2.1). Metabolite profiling by liquid chromatography mass spectrometry (LC-MS/MS) (249) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (250) have also been reported as alternative approaches. The discovery of uncultivable causal organisms in bacteriuria (179) emphasizes the need for new molecular approaches to aid not only diagnostics for the identification and management of infection, but also our understanding of the etiology of bacteriuria.
Conclusions and Future Directions

Bacteriuria is among the most globally prevalent infections and a massive financial burden on healthcare costs estimated at US$2.5-3.5 billion in medical expenses and societal costs annually in the USA alone (209, 210). Novel molecular tools will drive new discoveries in defining how bacteriuria progresses in different patients, and how contemporary culture-based diagnostics can be improved with next generation detection methods. Cost analyses will be important for new molecular methods to compare to current culture methods. Another crucial aspect (not addressed in this review) is the emerging need to quickly test not only for bacteriuria, but for antibiotic susceptibility. Questions addressed from a clinical viewpoint are better at defining the differences in ‘significant’ bacteriuria for different organisms in specific patient groups, and the implications for diagnostic approaches. Microbiologically, we need to better define the lifestyle adaptations that microbes use to aid bacteriuric potential. We also need to understand how ABU due to different bacteria interfaces with the host immune system, and how this occurs in different patient populations. Another unanswered question is how ABU due to organisms other than *E. coli* impact the incidence of sUTI. Finally, we need to investigate whether (and how) non-cultivable bacteria may modify the ability of cultivable causal organisms to cause infection, and whether ABU can be more effectively applied as a prophylactic probiotic approach to combat sUTI. Future studies will address these key questions.
Chapter 3

Molecular mechanisms of bacteriuria: promoting persistence through diversity

This chapter includes a co-authored paper currently in submission. The bibliographic details of the co-authored paper, including all authors, is:

Frontiers in Cellular and Infection Microbiology (in preparation).

My contribution to the paper involved:

Literature review, analyses of previously published data, and writing manuscript.

(Signed) _________________________________ (Date)______________

Deepak S. Ipe

(Countersigned) ___________________________ (Date)______________

Corresponding author of paper: Glen Charles Ulett

(Signed) _________________________________ (Date)______________

Supervisor: Glen Charles Ulett
Introduction

Bacteriuria and urinary tract infection

The term ‘Asymptomatic Bacteriuria’ (ABU or ASB) is effectively synonymous with asymptomatic Urinary Tract Infection (UTI) in defining the isolation of a specified semi-quantitative count of bacteria in an appropriately collected urine specimen from a person without signs or symptoms related to UTI (169, 170). Bacteriuria is used as a marker for symptomatic UTI (sUTI) and has been used historically to assist in grading the severity of infection. Establishment of bacteriuria in an individual depends on entry of an organism with bacteruric potential into the urinary tract. Bacteriuria can persist in an individual for many months or years, which may be related to an organism being sufficiently adapted to urine to survive in this unique and, naturally hostile host niche. Bacteruric potential in an organism not only encompasses microbial survival in urine but also enables the organism to grow (i.e. replicate) and re-grow, and endure host defense mechanisms that include dilution, voiding, frequent flushing (251, 252), and (as we will see) antimicrobial constituents. Bacteruric potential enables a microbes’ urinary survival despite several antimicrobial defense molecules that are normally present in urine and kill bacteria or inhibit their binding to urothelial cells. Examples of these defense molecules include the Tamm-Horsfall glycoprotein (also known as uromodulin), and P blood group antigen (253-255). The characteristics in microbes that can afford bacteruric potential thus provide means of influencing the progression and persistence of UTI. Key differences in microbial strategies for urine growth have been described, and may affect bacteriuria differently in different scenarios. In this review, we analyze bacterial traits known to support urine growth, and methods for modeling bacteriuria using synthetic human urine (SHU). We also propose a standardized SHU media for research applications focused on bacteriuria, and discuss related limitations.

Microbial bacteruric potential and host dynamics
Several recent reviews have focused on the pathogenesis and progression of acute and sUTI (141, 209, 210), and ABU (256, 257) and will not be revisited here. Instead, we will focus on the factors that influence bacteriuria: microbial bacteruric potential and host dynamics. It is important to note that the progression of bacteriuria depends on microbe traits as well as host factors. Hooton et al., reported that most individuals who suffer persistent UTI do not harbour the same strain of organism over time (173), implying that turnover of causal organisms is dynamic and that most ABU episodes are transient. Replacement of colonizing strains with other strains during the course of bacteriuria has been studied mostly for E. coli, the most common cause of ABU (256). For E. coli, long-term bacteriuria appears to select for attenuated virulence phenotypes of colonizing strains (211). While most microbes are killed by exposure to human urine (discussed further below), it is clear that some organisms have much higher bacteruric potential than others, which would effect strain replacement. Antibiotics halt the progression of bacteriuria and lower the incidence of infection (215); however, patients infected with E. coli experience re-colonization with the same or similar organism at high rates (196), reemphasizing the dynamic nature of causal organisms during bacteriuria and the role of host variables such as therapeutic intervention. Here, it is notable that treatment with antibiotics not recommended as routine for most individuals with ABU (258). The host factors most strongly associated with promotion of long-term bacteriuria and ABU are defects in innate immune signaling pathways such as the TLRs (259). Several recently discovered traits that effect the growth of microbes in urine, which is the focus of this review, may therefore influence the progression of bacteriuria equally or more so than host traits. Thus, bacteriuria is related to the colonizing microbes’ bacteruric potential, the characteristics’ of the host such as genetic immunodeficiency, re-current infection and strain replacement, and antibiotic therapy where administered.

**Microbial metabolism and growth fitness for urine**
Bacteriuria depends on a microbes’ survival of local host defense mechanisms including the antimicrobial properties of urine. Beyond survival, urine growth through metabolism can influence bacteriuria by maintaining a pool of colonizing organisms regardless of adherence to host cells, and urodynamic host properties such as urine flow rates and voiding that differ between individuals (260). Non-voided organisms in residual urine can grow and re-grow to maintain infection independent of host factors providing sufficient bacteruric potential is present. Discoveries using ABU microbes and their metabolic activities during urine growth have helped to shape our understanding of how bacteriuria progresses, and how it might interface with the host immune system. Metabolic mechanisms of urine growth have been studied in detail in ABU E. coli strain 83972, which displays robust fitness for urine growth (261, 262). Growth in urine is not, notably, a defining feature of all (or limited to) ABU E. coli, and has been reported in uropathogenic E. coli (UPEC) strains isolated from patients with acute sUTI (263-265). In fact, a recent study identified various ABU E. coli strains that grew no better in urine than many UPEC strains, showing that this fitness trait is not restricted to ABU organisms (266). Poor urine growth has been reported for several fecal E. coli isolates in other studies (263, 267). These differences exemplify the adaptability of phenotypes for urine growth among E. coli. In ABU E. coli 83972, urine growth has been investigated as a prophylactic means to prevent or treat acute UPEC sUTI (174-176, 212, 268, 269). The metabolic basis for urine growth of ABU E. coli 83972 appears to involve transport and degradation pathways for galacturonate, glucuronide and galactonate (261), and antioxidant defense mechanisms (266). For ABU E. coli and UPEC, details of the metabolic pathways activated in urine are available elsewhere (261, 268). The guaA and argC genes are critical for E. coli growth in urine. The guaA gene encodes guanosine monophosphate (GMP) synthase which catalyses the synthesis of GMP from xanthosine 5'-monophosphate (XMP) (270). argC encodes for N-acetylglutamylphosphate reductase which catalyses the synthesis of acetylglutamate semialdehyde from acetylglutamyl phosphate (270). A lack of urinary guanine (or
derivatives), combined with an inability of *E. coli* to synthesize these de novo, prevents the synthesis of other guanine (or derivative)-dependent products that are essential in urine (270). In a recent *E. coli* transposon mutagenesis study, *argC* and *carAB* mutants had reduced growth in urine confirming the previously identified role for arginine metabolism in *E. coli* growth in urine (271). The *carAB* operon, consisting of the two genes *carA* and *carB*, encodes carbamoylphosphate synthetase which synthesizes arginine and is important for pyrimidine metabolism (271).

Six bacterial species other than *E. coli* have been reported to grow in and/or metabolize constituents of human urine. These are: *Enterococcus faecalis* (272, 273), *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* (274), *Staphylococcus saprophyticus* (275) and *Streptococcus agalactiae* (276) (Table 3.1). In contrast to *E. coli*, however, little is known about the mechanism used by organisms other than *E. coli* for robust urine growth. Recent data on *E. faecalis* has revealed some similarities to the mechanisms used by *E. coli*. *E. faecalis* expresses multiple virulence genes during growth in urine (277) including genes for iron transport (273). Activation of iron utilization mechanisms was recently reported in urine growth assays with *E. coli* (278). Akin to urinary iron limitation as a ‘nutritional immunity’ host defence strategy, low concentrations of manganese were predicted to be important in limiting *E. faecalis* urine growth (273, 279, 280). In contrast to activation of pathways for galacturonate, glucuronide and galactonate as described for *E. coli* (261), *E. faecalis* activated citrate and aspartate metabolic pathways, and represses glucose uptake (273). This is a distinct mechanism from *E. coli* hinting at the existence of diverse mechanisms for urine utilization in Gram-negative and -positive organisms. It is known that human urine contains more citrate than glucose (281-283), which could promote the growth of *E. faecalis*. *E. faecalis* also upregulates genes for utilization of sucrose (and perhaps fructose), another constituent of urine (281, 284).
### Table 3.1: Summary of known mechanisms that contribute to bacteruric potential in microbes

<table>
<thead>
<tr>
<th>Traits that confer bacteruric potential</th>
<th>Bacterial species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ability to utilize human urine as a substrate for growth</td>
<td><em>E. coli</em></td>
<td>(212, 268, 269), many others</td>
</tr>
<tr>
<td></td>
<td><em>E. faecalis</em></td>
<td>(272, 273, 277)</td>
</tr>
<tr>
<td></td>
<td><em>P. vulgaris</em></td>
<td>(285)</td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em></td>
<td>(274)</td>
</tr>
<tr>
<td></td>
<td><em>K. pneumoniae</em></td>
<td>(274)</td>
</tr>
<tr>
<td></td>
<td><em>S. saprophyticus</em></td>
<td>(275)</td>
</tr>
<tr>
<td></td>
<td><em>S. agalactiae</em></td>
<td>(276)</td>
</tr>
<tr>
<td>Tolerance to high levels of D-serine</td>
<td><em>S. saprophyticus</em></td>
<td>(275)</td>
</tr>
<tr>
<td>Capacity for synthesis of guanine-dependent products critical for survival in urine</td>
<td><em>E. coli</em></td>
<td>(270)</td>
</tr>
<tr>
<td>Expression of iron acquisition systems for growth in nutrient limiting environment</td>
<td><em>E. coli</em></td>
<td>(278)</td>
</tr>
<tr>
<td></td>
<td><em>E. faecalis</em></td>
<td>(273)</td>
</tr>
<tr>
<td>Osmoadaptation, and intracellular accumulation of glycine betaine</td>
<td><em>E. coli</em></td>
<td>(286, 287)</td>
</tr>
<tr>
<td>Malic acid metabolism</td>
<td><em>S. agalactiae</em></td>
<td>(276)</td>
</tr>
</tbody>
</table>
Other *E. faecalis* genes thought to function in urine growth include those related to import of phosphorylated sugars and glycerol, N-acetyl glucosamine metabolism (273), cysteine synthase, and pathways for conversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate. This suggests that urinary aspartate may be used for nitrogen metabolism (288). Finally, while *E. faecalis* is auxotrophic for multiple amino acids, human urine contains several including arginine, glutamate, glycine, and leucine (281, 288).

Recently, phenotype metabolic array fingerprinting was described for another Gram-positive organism with bacteruric potential for urine growth, ABU *S. agalactiae* (276). Comparison with uropathogenic strains of the same organism that were unable to grow in urine revealed that malic acid catabolism was important for this organism's ability to grow in urine. Malic acid utilization is related to the malic enzyme metabolic pathway that catalyzes the oxidative decarboxylation of malate (a frequent component of normal human urine) to pyruvate and CO\(_2\). This underpins malolactic fermentation that is typically associated with wine deacidification (289).

**Surviving urine three ways: resistance, acquisition and osmoadaption**

Urine is naturally antiseptic; hypertonicity with low pH and high concentrations of urea inhibit most bacteria, which aids their elimination from the bladder (286). Nitrite in mildly acidified urine inhibits the growth of common Gram-negative uropathogens (290), and other abundant urinary proteins such as Tamm-Horsfall protein (THP) are antimicrobial (291, 292). Some bacteria with bacteruric potential have augmented a capacity for urine growth with resistance to urine antiseptic properties, as summarized in Table 3.1. For example, *S. saprophyticus* tolerates high concentrations of D-serine, which is inhibitory for bacteria that lack a D-serine deaminase. This enables *S. saprophyticus* to
survive in urine (275). Iron limitation is another key antimicrobial part of urine. Here, the presence iron chelators such as lactoferrin (293) compounds the problem of nutritional immunity for microbes (294). This may be amplified during states of increased production of iron chelators such as during certain diseases (295, 296). Iron acquisition systems (i.e. siderophores) offer bacteruric potential to E. coli (264, 278, 297-300). Homologs are upregulated in E. faecalis during urine growth (273). Siderophores that compete with host chelators have been shown to act as favorable ligands for cations other than iron, such as copper, which may offer bacteruric potential (301). In terms of other antimicrobial constituents in urine, resistance to THP has not been described to date, and variable resistance to other soluble urinary defense molecules such as nitrite and ascorbic acid could provide bacteruric potential. However, little is known about this facet beyond the direct effects of these on the chemical properties of urine (290). Finally, many organisms are probably unable to survive the oxygen concentrations encountered in the bladder (302, 303).

Urea is abundant in urine and is antibacterial (304). Key osmoadaptive systems enable some bacteria to survive the stressful hypertonic conditions of urine. Bacterial accumulation of osmotically compatible solutes that are present in urine (e.g. betaines) provides vital bacteruric potential by effectively enabling microbes to resist dehydration (286, 287). Osmoadaptive systems respond to changes in tonicity in urine, and support survival by counteracting low pH, high urea concentrations and hypertonicity (286). For E. coli, glycine betaine is a central osmoprotectant to resist urea toxicity, and its accumulation in the cell is essential to mechanistic, adaptive responses to osmotic stress (286, 305). E. coli increases the activity of potassium transport systems that encompass TrkG, TrkH, and Kup (306), or Kdp (307) to counteract osmotic stress. Other systems involve trehalose as an organic osmolyte; its induction is triggered in conditions of high potassium (and glutamate) levels (308), and its accumulation elicits
the release of potassium from the bacterial cell (309). This might also aid growth since trehalose can be used as a carbon source in the presence osmotic stress (310, 311). OmpR regulates osmoadaptive genes in E. coli (312). Osmotic stress also suppresses the expression of fimbriae and flagellin (313, 314). Future studies of non-E. coli bacteria that grow in urine will be important to identify additional mechanisms of resistance to urinary antimicrobial properties, nutrient acquisition and osmoadaption.

The interface between bacteriuria and immune surveillance

Inflammation is a critical part of sUTI pathogenesis (141, 210) and was recently shown to involve >2500 host genes that drive antibacterial responses within just hours of UPEC colonization of bladder (88, 315). Inflammation in response to ABU can encompass pyuria, cytokine release (IL-1α, IL-6, IL-8), and antibody production, which has been documented in elderly adults, as reviewed elsewhere (316). The benign and minimally inflammatory nature of these responses in ABU are reflected in the absence of morbidity in ABU individuals who do not receive therapy (316-318). Details of the way in which ABU bacteria induce inflammatory responses, or minimize them, are now emerging and offer insight into how microbes may modulate host defense strategies to promote persistent bacteriuria. In contrast to ABU that may persist with minimal immune activation, excessive inflammation may contribute to chronic sUTI (319). Certain acute sUTI symptoms have also been linked to specific inflammatory events (320). ABU E. coli minimizes inflammation by averting adherence due to a lack of fimbriae expression; this limits immune activation (261) and results in long-term ABU (321, 322). Hernandez et al., showed that ABU E. coli activates IFN3 and TLR4-dependent signaling, however, triggering a response that depends on the genetic background of the host (323). TLR4 senses P-fimbriated E. coli (324), and TLR4 mutations may favor ABU by impeding innate responses (325). This also raises the
question of whether ABU may influence subsequent encounter(s) with other uropathogens. A recent study on streptococcal UTI showed an influence on the severity of subsequent *E. coli* UTI in mice (326). This implies that immune activation triggered by ABU might affect subsequent development of acute or chronic sUTI by diverse and potentially pathogenic bacteria. Overall, these data align with clinical data where patients with *E. coli* ABU suffer re-colonization at high rates following therapy (196). Important studies for the future will be to compare ABU innate immune induction due to non-*E. coli* organisms to define the nature of these host responses, and the way in which different microbes effect them.

**Modeling bacteriuria in vitro: Synthetic Human Urine (SHU)**

Urine is unique from a microbial perspective and its chemical makeup, distinct from all other bodily fluids, has been modeled for studying the growth of microbes for 50 years (327). Urine has a low pH (averaging ~6.0) and a high osmolality due to the presence of salts and urea (328, 329). The peptides, proteins, and organic acids present may be utilized by microbes for metabolism (330). Urine is dynamic in flow rate and composition, which changes subject to diet, age, gender and health status and disease. Decreased levels of THP, for example, are associated with diabetes (331) and UTI (197). The chemical makeup of urine affords antiseptic properties (329) that kill or inhibit most urogenital tract commensals (286). Most data on the microbial traits that afford bacteruric potential have been derived from studies of synthetic human urine (SHU). Analysis of bacterial growth in SHU has led to discoveries of bacteruric potential, and refinements in chemical composition have been used for different types of microbes. In total, eight original SHU media were described between 1976 and 2010, and have provided the basis for all subsequent SHU research. These are summarized according to research application in Table 3.2. Few studies have modified
SHU recipes for explicit applications, and all current studies stem from the original SHU media descriptions listed in Table 3.2.

The use of SHU avoids the issue of variable chemical composition encountered with fresh human urine; variance in urinary constituents between individuals, such as the indefinite pyrophosphate matrix makeup, and the presence or absence of dozens of compounds in the healthy adult population (281) is a challenge for standardizing research. Methods for ‘normalizing’ fresh human urine include pooling samples and/or adjusting dilution/concentration according to creatinine concentration, specific gravity, and osmolality. The most widely used method is creatinine adjustment (332), however no bacteriuria research studies to date have applied these methods for normalizing, and the effects on data interpretation are untested. Volume limits are usually not an issue but have been difficult for some studies (333). As a surrogate model, the benefits of SHU are largely defined by how closely it can reflect the chemical complexity of fresh human urine. Urine from a healthy adult contains glucose (0.2-0.6 mM) (283), creatine (10-20 mM) (332), and glycine with low levels of other amino acids such as histidine, glutamine, methionine, proline, glutamate, arginine, cysteine and branched chain amino acids (273, 288). It contains trace fatty acids, citrate (1-2 mM) (282), sucrose (70-200 μM) (284), and manganese (nM range) (279). To standardize SHU composition for bacteriuria studies, we have formulated ‘composite SHU media’ based on the studies described above, and have compared this to the most thorough prior studies of ‘typical human urine’ (281, 334). This composite SHU media is described in Table 3.3, shown alongside specific constituents and their concentrations. The most noteworthy supplement to standard SHU from physiology and cell biology studies (335), for specific use in bacteriuria applications, is the necessary addition of the 5% Lysogeny Broth (LB) for Gram-negative bacteria, or 5% Todd-Hewitt Broth (THB) for Gram-positive bacteria. Dextrose supplementation has been used for fungal UTI
studies such as for Candida sp. (336). This composite SHU media for infection studies is easily prepared, inexpensive, chemically stable, and applicable to most UTI organisms. It is important to highlight a goal for future studies however, aimed at further refining SHU for chemical definition in the absence of LB and THB.
Table 3.2: Summary of original and subsequent studies that have used chemically-defined Synthetic Human Urine (SHU) media for research applications

*Reference(s) included in bibliography of original study (minimal recipe details in reference citing the original study); †Cited by: these references cited the immediate preceding reference in the list rather than the original study. ‡Original study of (367) cited by (368) does not provide a SHU recipe. §Original studies of (339) and (353) cited by (352) provide a SHU recipe.

<table>
<thead>
<tr>
<th>Original Study</th>
<th>Category</th>
<th>Specific Application</th>
<th>Microbes or Cells Used</th>
<th>Relevance</th>
<th>Reference(s) Citing the Original Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>(337)</td>
<td>Physiology</td>
<td>Study of calcium oxalate monohydrate precipitation</td>
<td>N/A</td>
<td>Urolithiasis</td>
<td>(338)</td>
</tr>
<tr>
<td>(339)</td>
<td>Infection</td>
<td>Development of SHU to study urinary pathogens</td>
<td>E. coli, P. aeruginosa, P. mirabilis, S. epidermidis</td>
<td>UTI</td>
<td>(340)</td>
</tr>
<tr>
<td>(341)</td>
<td>Physiology</td>
<td>Development of standard reference SHU for study of in vitro urolithiasis assays</td>
<td>N/A</td>
<td>Urolithiasis</td>
<td>(338, 342-345) *(346-351)</td>
</tr>
<tr>
<td>(352)</td>
<td>Cell Biology</td>
<td>Comparisons of multiple SHU media &amp; study in epithelial cell culture assay</td>
<td>Kidney epithelial cells</td>
<td></td>
<td>*(338, 339, 342-344, 353)§</td>
</tr>
<tr>
<td>(354)</td>
<td>Infection, &amp; Physiology</td>
<td>Investigation of infection-induced urinary stones</td>
<td>E. coli, P. aeruginosa, P. mirabilis, Proteus rettgeri, Providencia stuartii, Morganella morganii, Klebsiella oxytoca, Citrobacter koseri,</td>
<td>UTI</td>
<td>Urolithiasis (355-357) (358) *(361) Cited by: (336, 359, 360) and (361) Cited by: (362) *(363)</td>
</tr>
<tr>
<td>(364)</td>
<td>Infection</td>
<td>Study of the efficacy of gentamicin in SHU</td>
<td>E. coli, P. aeruginosa</td>
<td>UTI</td>
<td>(365)</td>
</tr>
<tr>
<td>(334)</td>
<td>Physiology</td>
<td>Characterization of urine</td>
<td>N/A</td>
<td>All studies</td>
<td>No citations received</td>
</tr>
<tr>
<td>(350)§</td>
<td>Physiology</td>
<td>Study of calcium oxalate crystallization</td>
<td>N/A</td>
<td>Urolithiasis</td>
<td>(366)</td>
</tr>
</tbody>
</table>
Table 3.3: Synthetic Human Urine (SHU) constituents, and composite SHU media recipe proposed for infection related studies

<table>
<thead>
<tr>
<th>Component</th>
<th>Defined in (n)</th>
<th>Mean Conc. (Range)</th>
<th>Composite SHU</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8/8</td>
<td>113.3 (54-231) mM</td>
<td>100 mM</td>
<td>(335, 337, 339, 341, 350, 352, 354, 364)</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>8/8</td>
<td>17.0 (9.0-155,800) mM</td>
<td>17.0 mM</td>
<td>(335, 337, 339, 341, 350, 352, 354, 364)</td>
</tr>
<tr>
<td>pH</td>
<td>7/8</td>
<td>6.1 (5.7-7.2)</td>
<td>6.2</td>
<td>(335, 339, 341, 350, 352, 354, 364)</td>
</tr>
<tr>
<td>Urea</td>
<td>6/8</td>
<td>281 (170-500) mM</td>
<td>280 mM</td>
<td>(337, 339, 350, 352, 354, 364)</td>
</tr>
<tr>
<td>KCl</td>
<td>6/8</td>
<td>58.5 (21.5-162.7) mM</td>
<td>38.0 mM</td>
<td>(335, 337, 341, 352, 354, 364)</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>6/8</td>
<td>5.3 (2.5-12.0) mM</td>
<td>4.0 mM</td>
<td>(335, 339, 341, 352, 354, 364)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>5/8</td>
<td>8.7 (4.0-13.2) mM</td>
<td>9.0 mM</td>
<td>(337, 339, 352, 354, 364)</td>
</tr>
<tr>
<td>Na₃C₆H₅O₇</td>
<td>5/8</td>
<td>3.4 (2.2-5.0) mM</td>
<td>3.4 mM</td>
<td>(335, 341, 352, 354, 364)</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>5/8</td>
<td>36.6 (15.0-86.8) mM</td>
<td>20.0 mM</td>
<td>(335, 339, 352, 354, 364)</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>5/8</td>
<td>3.2 (2.0-5.9) mM</td>
<td>3.2 mM</td>
<td>(335, 337, 339, 341, 352)</td>
</tr>
<tr>
<td>Na₂C₂O₄ (Na Oxalate)</td>
<td>5/8</td>
<td>0.38 (0.1-1.2) mM</td>
<td>0.18 mM</td>
<td>(341, 352, 354, 364)</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>4/8</td>
<td>20.9 (3.6-43.6) mM</td>
<td>3.6 mM</td>
<td>(335, 337, 341, 352)</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>4/8</td>
<td>6.5 (6.1-186,800) mM</td>
<td>6.5 mM</td>
<td>(335, 337, 350, 352)</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3/8</td>
<td>16.0 (7.0-20.6) mM</td>
<td>16.0 mM</td>
<td>(339, 354, 364)</td>
</tr>
<tr>
<td>Chemical</td>
<td>Concentration</td>
<td>Comment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
<td>---------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C$_3$H$_4$N$_2$O$_3$</td>
<td>0.6 (0.4-1.0) mM</td>
<td>0.6 mM (337, 339, 352)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>13.5 (2.0-25.0) mM</td>
<td>13.5 mM (339, 352)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgCl$_2$·6H$_2$O</td>
<td>3.2 (3.2) mM</td>
<td>3.2 mM (354, 364)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osmolality</td>
<td>586 (446-725) (mOsm/kg)</td>
<td>600 (mOsm/kg) (352, 364)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C$_3$H$_6$O$_7$</td>
<td>1.7 (1.4-2.0) mM</td>
<td>- (337, 339)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH$_3$OH</td>
<td>17.1 mM</td>
<td>- (337)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C$_3$H$_5$NO$_3$</td>
<td>2.8 mM</td>
<td>- (337)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>7.0 mM</td>
<td>- (339)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C$_3$H$_6$O$_3$</td>
<td>1.1 mM</td>
<td>1.1 mM (339)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeSO$_4$·7H$_2$O</td>
<td>0.005 mM</td>
<td>0.005 mM (339)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K$_3$C$_6$H$_5$O$_7$ (K citrate)</td>
<td>2752000 mM</td>
<td>- (350)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg(NO$_3$)$_2$</td>
<td>2.5 mM</td>
<td>- (350)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptone</td>
<td>0.1% (w/v)</td>
<td>0.1% (w/v) (339)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.0005% (w/v)</td>
<td>0.0005% (w/v) (339)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LB*</td>
<td>3.5% (2.5-5%) (w/v)</td>
<td>5% (w/v) (336, 358-360, 369)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>THB*</td>
<td>-</td>
<td>2.5% (w/v) [Gram +ve Only] (276)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Component</td>
<td>SHU Composition</td>
<td>Concentration</td>
<td>References</td>
<td></td>
</tr>
<tr>
<td>--------------------</td>
<td>-----------------</td>
<td>---------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>Dextrose</td>
<td>1/8</td>
<td>2.0% (w/v)</td>
<td>(336)</td>
<td></td>
</tr>
</tbody>
</table>

*Indicates the number of original studies that defined the component in their SHU Media Recipe; †Refers to Average Concentration, and (Concentration Range); ‡Proposed Composite SHU Media Concentrations are chosen based on average values in referenced studies, and most compare closely to the typical human urine chemical composition as reported in (334) [e.g. NaCl 137 mM, Urea 223 mM, KCl 22 mM, Creatinine 13.3 mM, MgSO₄ 6.5 mM]. In some cases (e.g. Na₂HPO₄, Na₂SO₄, KCl, Na₂C₂O₄) the means and composite SHU values have been formulated to exclude extreme upper range values from (335, 350). §References cited for recipe components are only original studies, except for LB, THB, and dextrose, for which all published studies using these components are cited.
In addition, even the proposed composite SHU excludes a range of natural constituents of human urine such as hormones, iron chelators, and pyrophosphates that could influence microbial growth. As a balance between logistics, economy, and stability/feasibility, the proposed composite SHU should be of value for future bacteriuria studies.

**Conclusions and Future Directions**

A microorganisms capacity for urine growth may aid in establishing long-term bacteriuria and is relevant to many causal species. New discoveries on immune activation by ABU shows that this form of asymptomatic infection does not exist entirely under the radar of immune surveillance, as previously thought. Continued use of SHU and applied molecular tools will drive new discoveries in our understanding how bacteriuria progresses in the host, and how contemporary diagnostics can be improved with next generation detection methods. There are some questions that can be addressed now to deliver some of these key advances in our knowledge of bacteriuria. From the clinical viewpoint, this includes defining differences in ‘significant’ bacteriuria for different organisms among specific patient groups such as healthy adults, diabetic and institutionalized elderly individuals, and the implications for diagnostic methods. Microbiologically, we need to define the lifestyle adaptations, other than those described for D-serine, guanine and iron acquisition that microbes use to aid bacteruric potential, and define the molecular basis of urine growth in non-*E. coli* ABU organisms. Other questions include how ABU interfaces with host immune mechanisms for different organisms, and does this occur equally amongst different patient populations? What are the states of ABU that impact on the incidence and severity of subsequent sUTI due uropathogens other than *E. coli*? Finally, how effective might ABU be as a prophylactic probiotic approach to lower the incidence of sUTI? Future studies will address these and other key questions in the field.
Chapter 4

Investigation of growth of asymptomatic and uropathogenic Streptococcus agalactiae in normal human urine in vitro

This chapter includes a co-authored paper. The bibliographic status of the co-authored paper, including all authors, is:

Infect. Immun. 80:3145-60.

Supplementary figures in Appendix 2.

My contribution to the paper involved:

Design and generation of data for all growth experiments (urine and THB), serotyping, clinical description of isolates, analysis of the experimental results, making of all graphs, and writing of the manuscript.

(Signed) _________________________________ (Date)________________

Deepak S. Ipe

(Countersigned) _________________________________ (Date)________________

Corresponding author of paper: Glen Charles Ulett

(Countersigned) _________________________________ (Date)________________

Supervisor: Glen Charles Ulett
Introduction

*Streptococcus agalactiae* – a human pathogen

*Streptococcus agalactiae* is also known as Group B *streptococcus* (GBS) is a Gram-positive, β-hemolytic, chain-forming bacterium and a commensal within the genital tract flora in approximately 25% of healthy adult women (5). Studies on neonatal infections revealed that this Gram-positive pathogen is a leading cause of life threatening infections in newborns, pregnant women (3, 151, 370), elderly and immunocompromised individuals (12, 371). In neonates, most common infections include meningitis, sepsis and pneumonia (372-374). *S. agalactiae* infection in neonates initially occurs when bacteria ascends from vagina to the amniotic fluid and these bacteria can be get into the fetal lungs, which in turn can lead to bacteremia. When newborn pass through the birth canal also *S. agalactiae* can cause infection, mainly colonizing at mucus membrane sites in the gastrointestinal or respiratory tracts (375, 376). In early 1970s, the mortality rate reported was 55% for those neonates with invasive *S. agalactiae* infection (377, 378). This organism is also responsible for the maternal cervicovaginal colonization via vertical transmission of bacteria from mothers to their infants (379). Growth of the bacteria in human amniotic fluid is believed to be associated with an increased risk for infection in utero. *S. agalactiae* have shown rapid growth in human aminiotic fluid with a generation time of ~35min in logarithmic phase with bacterial density reaching $10^9$ CFU/ml in stationary phase (380).

In addition to the above mentioned infections, *S. agalactiae* can also cause urinary tract infections (UTIs), (381). The spectrum of *S. agalactiae* UTI includes asymptomatic bacteriuria (ABU), cystitis, pyelonephritis, urethritis, and urosepsis (16, 34, 36, 43). In one study *S. agalactiae* was in found 26% of cases of ABU in diabetes gravidas (382). Bacteriuria of any count due to *S. agalactiae* in pregnancy is routinely treated with
antibiotics to minimize the risk of vertical transmission to the neonate (190, 256). A study conducted in a perinatal centre in India showed that among 11060 live born blood cultures, *S. agalactiae* was isolated from 10 babies with two of them asymptomatic (383).

Bacterial adherence to bladder tissue is generally considered to be the first step in colonisation, enables the bacteria to survive in urinary tract (261). However, another fitness traits that contribute to the persistence of some bacteria, such as ABU *Escherichia coli*, in the urinary tract is the ability to grow in human urine. This enables uropathogens such as *E. coli* to persist in the host by re-growth of non-voided organisms in residual urine, and offers a competitive advantage independent of urothelial cell binding and inflammation (261, 262). Other organisms have also been reported to have the ability to grow in human urine including staphylococci (275) and enterococci (263, 273). For this, bacteria must withstand low pH, high salt levels, nutrient limitation, mildly acidified urine, and exposure to antimicrobial proteins secreted in urine, such as Tamm-Horsfall protein. Studies on metabolic basis for urine growth revealed transportation and degradation pathways for galacturonate, glucuronide and galactonate in ABU *E. coli* (261). Optimal growth of uropathogens in human urine also dependent on synthesis of guanine, arginine, and glutamine (384). Antioxidant defense mechanisms have also been known as being important for urine growth of *E. coli* (266). Russo et al., demonstrated that synthesis of guanine-dependent products was critical for survival and growth of *E. coli* in urine (270). In another study, arginine metabolism was shown to play an important role in growth of *E. coli* (271). Tolerance to high levels of, or requirement for, serine has been linked to survival of *Staphylococcus saprophyticus* (275) and *E. coli* in urine (384), respectively. In addition, iron acquisition has been identified as potentially important in *E. coli* (278) and *E. faecalis* (273) for urine growth. Russo T. A. et al., demonstrated that uropathogenic
bacteria also possess specific trait to enable growth and survival in human urine, which may influence UTI. They have recognized two genes named guaA and argC, which have been demonstrated to contribute to the growth of uropathogenic *E. coli* in urine in vitro and both have diminished urovirulence *in vivo* (270). Uropathogenic bacteria grow well in human urine whereas non-uropathogens would not be able to survive (263). Robust growth of uropathogenic *E. coli* (UPEC) in urine can even permit colonisation in the absence of specific adherence organelles. So, ability of UPEC to survive and efficiently utilize human urine is an important adaptation for inhabiting the urinary tract (267).

However, fitness of *S. agalactiae* for growth in human urine has not been investigated previously. This study will be focusing on the ability of asymptomatic and uropathogenic *S. agalactiae* (358 isolates in total) to utilize the human urine as a growth medium and identify the potential phenotypical differences between the isolates from different clinical conditions to grow in human urine. Also define the serotype distribution among the selected isolates cultured from different *S. agalactiae* UTI conditions.
**Materials and methods**

A total of 358 *S. agalactiae* isolates were included in this study and all strains were isolated from individuals with single-organism UTI as described elsewhere (16). For each isolate detailed clinical records such as date admitted, sex, age, counts of *S. agalactiae* etc., were available and these were used to group isolates according to disease presentation. Isolates from three patient groups were used for this study: (1) cystitis isolates, i.e. symptomatic patients who presented with acute UTI, >10⁴ CFU/ml, (2) low-grade ABU isolates, i.e. from asymptomatic patients with <10⁴ CFU/ml, and (3) high-grade ABU isolates, i.e. from asymptomatic patients with >10⁴ CFU/ml. All isolates were also identified through capsular serotyping (latex agglutination (67)) and molecular serotyping (73) to elucidate which serotypes cause disease (16). For long time storage purpose, all *S. agalactiae* isolates were stored at -80°C in 25% glycerol and Todd-Hewitt Broth (THB).

Isolates were generally grown overnight on tryptone soya agar (TSA) containing 5% defibrinated horse blood or in Todd-Hewitt Broth (THB) at 37°C with or without shaking at 200rpm in Thermo scientific MAXQ 6000 incubator. For growth assays, isolates from overnight THB cultures were initially diluted (1:1000) in 1XPBS and then seeded (1:100) into filter-sterilised (with 0.45µM, Millipore-SLHV033RS), pooled human urine. Required urine for each assays were collected freshly from at least six healthy men and women volunteers who had no recent history of UTI or antibiotic usage (preceding month). In order to ease the filtration process with 0.45µM filter and to remove larger particles, the pooled urine been filtered through a bigger pore size filter system prior to the use of 0.45µM Millipore filter. For this, Buchner vacuum flask vacuum filtration method with the aid of Millipore glass filter assembly (Millipore-XX1004700) and a bigger pore sized, 47mm filter paper (Filtech-0282-047) been used. Fresh urine was
used within 48h of collection and sterilisation. This study was performed with approval from and in accordance with the guidelines of the Griffith University Human Research Ethics Committee (MSC/11/10/HREC).

For growth experiments, duplicate cultures were grown in flat-bottom 96 well plates (SARSTEDT-82.1581.001) at 37°C with shaking (200rpm). Thermo scientific MAXQ 6000 incubator was used for all cultures and growth assays. Optical density at 600nm (OD600nm) was read between 0-72h (2h intervals until 16h, then at 24h and 12h intervals thereafter) using POLARstar Omega BMG Labtech plate reader. To determine the precise number of viable bacteria over time and to correlate with OD600nm, colony counts were performed at 6h and 12h and every 12h thereafter upto 72h and bacterial number in CFU/ml was calculated retrospectively. To get the countable number of colonies 1:10 serial dilutions were performed in 1XPBS upto 10^-6. Then 5µl of each diluted samples including neat (not diluted) were spotted on to 5% horse blood TSA agar, air dried and incubated at 37°C, overnight. Colonies on the plates were counted next day and analysed the data by plotting the graphs in excel. Growth assays were repeated at least three to five times in independent experiments using different batches of pooled human urine. Separate replicate 96 well plates were maintained for colony counts and OD600nm readings. Growth assays in THB were also performed simultaneously, same way in 96 well plates as urine growth assays and OD600nm readings were recorded. All selected isolates (40 and 25) were also serotyped using Streptococcus antisera for precipitation (Group B type antisera) of Neufeld test from Statens Serum Institut, Denmark (385).
Results

Screening of 358 *S. agalactiae* isolates from three different patient groups (1) cystitis or acute UTI, (2) low-grade ABU and (3) high-grade ABU demonstrated a spectrum of fitness for growth in pooled human urine over a 72h period. To determine the precise number of viable bacteria over time colony counts were performed along with the turbidity measurement at OD600nm. For comparison studies and to elucidate any phenotype differences between the isolates from different patient groups, best fast growers and slow growers were selected. The data of colony counts (Fig 4.1 & Fig 4.2) and absorbance readings at OD600nm (Fig 4.3 & Fig 4.4) of all 358 isolates allowed to select best fast and slow growers in human urine. In total, among the 358 *S. agalactiae* isolates screened for fitness for growth in human urine, 40 fast growers and 25 slow growers were identified. Figure 4.1 and figure 4.3 depicting colony counts and OD600nm respectively of ten fast growers out of 40 selected from human urine growth. Colony counts and OD600nm of the rest of the 30 fast growers are showed in appendix 2, supplementary figures S1a-S1c & S3a-S3c respectively. Ten isolates from the selected 25 slow growers showed in figure 4.2 (colony counts) and figure 4.4 (OD600nm). Rest 15 of the 25 slow growers are demonstrated in appendix 2, supplementary figures, S2a-S2b (colony counts) & S4a-S4b (OD600nm).

All selected isolates from human urine growth experiments, including slow growers, grew well in nutrient rich media, THB (Fig 4.5a & 4.5b). This suggests that there is no general growth defect in any of the isolates. For example, isolate 807 in figure 4.2 (colony count) and figure 4.4 (OD600nm) demonstrating very slow growth in human urine whereas in THB, figure 4.5b, isolate 807 reached to an OD600nm of 0.55 by 24h. Thus this data suggest that those growth patterns demonstrated by these selected isolates are urine specific. This divergent phenotype in growth in human urine may be
because these fast growers possess or express particular gene/s which is responsible for utilizing available energy source from nutrient limited media such as human urine. The 40 fast growers were selected from the three patient groups as follows: acute UTI: n=0, low-grade ABU: n=2, and high-grade ABU: n=38. This compared to a distribution of 7 acute UTI isolates, 4 low-grade ABU, and 14 high-grade ABU isolates among 25 slow growers. Analysis of this distribution showed that individuals with high-grade ABU were significantly more likely to be colonized with a fast grower compared to patients with cystitis or low-grade ABU. Furthermore, 95% of fast growers in human urine represents high-grade ABU group, suggesting that patients with high-grade ABU are more adapted to grow in human thus extended survival in bladder. Serotyping results based on the Neufeld method of type-specific antisera (Fig 4.6) demonstrated that, among the 40 fast growers in human urine, types III (35%), Ia (15%) and V (12.5%) were predominant.
Fig 4.1: Colony Count of Fast Growers in Human Urine (out of 40 selected fast growers).

Fig 4.2: Colony Count of Slow Growers in Human Urine (out of selected 25 slow growers) in sterile pooled human urine (0h to 72h); identified from screening a total of 358 S. agalactiae isolates for fitness for growth in human urine.
Fig 4.3: Absorbance Reading at OD600nm of Ten Representative Fast Growing *S. agalactiae* Isolates (out of 40 selected fast growers).

Fig 4.4: Absorbance Reading of Ten Selected Slow Growing Isolates (out of selected 25 slow growers) in sterile pooled human urine (0h to 72h).
Fig 4.5: Absorbance Reading in THB

(a) Absorbance reading at OD600nm of ten representative urine fast growers (out of 40 selected fast growers) and (b) ten urine slow growers (out of selected 25 Isolates) in THB. Demonstrating there is no general growth defect.
Fig 4.6a: Isolate 91, agglutinating with Type II Group B antisera

Fig 4.6b: Isolate 91, not agglutinating with other types of antisera

**Fig 4.6: Serotyping** (a) Serotyping of isolate 91 demonstrated strong agglutination with Serotype II of Group B antisera but (b) not agglutinating with any other types of antisera (Type Ia, Ib, III-IX).
Discussion

Although we know several uropathogens such as *E. coli*, staphylococci and enterococci are capable of growing in human urine, our understanding of whether *S. agalactiae* can utilize human urine for growth is unknown. The main finding of this study is that some *S. agalactiae* strains, especially those associated with ABU, can utilize human urine for growth. It is important to note that the capabilities to utilize available energy sources from a nutrient limited media such as urine are different from one isolate to another, and our data shows this is true for *S. agalactiae*. Our study suggests that ABU isolates can grow better in human urine compared to uropathogenic isolates. Similar data has been reported for *E. coli*. For example, the ABU *E. coli* 83972 isolate grows well in human urine with a doubling time ranging from 45min to 60min when compared to other well-known UPEC isolates that were shown to exhibit a significantly slower growth rate (261). Overall, relatively few studies have addressed growth of other urinary pathogens in human urine. Importantly, a growth ability of *S. agalactiae* in human urine never been reported prior to this work and this may be one of the most important factors of *S. agalactiae* persistence and colonization in the human urinary tract.

In this study, we mainly focused on the characterisation of growth of a total of 358 *S. agalactiae* strains isolated from three different patient groups; (1) cystitis or acute UTI, (2) low-grade ABU and (3) high-grade ABU. Using this approach, we aimed to elucidate whether low-grade or high-grade ABU isolates might utilize urine for growth better compared to uropathogenic *S. agalactiae* isolates. The large number (358) of *S. agalactiae* isolates included in this growth study is a strength to this work and highlighted for the first time that *S. agalactiae* (mainly ABU *S. agalactiae*) can utilize
human urine readily; a similar finding compared to other urinary pathogens such as *E. coli* and staphylococci.

In order to elucidate whether isolates from different patient groups would show different growth patterns in human urine, colony counts and absorbance reading at OD600nm were measured to infer bacterial growth fitness. Viable bacterial counts quantified by colony counts technique were comparatively more reliable because of the variabilities of OD600nm readings of bacteria in human urine such as chain length, structure of the bacterial cell and cell size, clumping, and death of bacterial cells over time. However, absorbance readings were easy to record even in 2h intervals and were a useful guide to interpret the progress of growth of these bacteria over time. In this way, both techniques provided advantages for initial growth characterisation. Together, the results of both colony count and absorbance data vastly helped to characterise the most representative and reproducible fast growers (40 isolates) and slow growers (25 isolates) in human urine. Interestingly, among the 40 fast growers, the majority (95%) belonged to the high-grade ABU group. This is a significant finding and strongly supports the notion that ABU isolates have superior growth ability in urine over uropathogenic isolates in the clinical setting (271, 386).

Human urine is a very complex growth medium and its chemical composition, pH and osmolality widely varies depending on the dietary intake of a person. Urine contains metabolic breakdown products from various foods and drinks. This biofluid consist of high concentrations of urea, inorganic salts, creatinine, ammonia, organic salts, various toxins and antimicrobial products and pigmented products such as urobilin which gives urine its characteristic colour. This includes traces of iron and other metabolites and organic acids such as malic acid, lactic acid, arginine, and amino acids like glycine,
leucine, aspartic acid, and glutamine (281). Even though urine is composed of all these chemical compounds, sugars and metabolites, it also possesses antibacterial activity to some extent which is correlated with osmolality and pH. Antimicrobial activity of a urine sample with a lower pH is much higher than urine sample with a pH range between 6.0 and 7.0 (263). High urea concentrations are also considered to be a strong inhibitory agent for several bacteria including for *E. coli* (387, 388).

Bacterial strains like ABU *S. agalactiae* which are capable to survive the normally inhibitory environment of urine, and utilize available energy source in human urine must therefore possess or express specific genes that confer an ability to overcome these natural inhibitory activities of human urine. Furthermore, specific metal ions, sugars, or other organic acids might be used by *S. agalactiae* as an energy source to grow in human urine. The growth experiments we conducted in human urine clearly demonstrate that the majority of ABU isolates can readily use human urine for growth. This data also indicates that there is a difference between high-grade ABU isolates and acute UTI isolates in utilization of components in human urine for growth. Serotyping of selected *S. agalactiae* isolates from the three different groups were also performed and demonstrated interesting results. Here, the majority of fast growers were of serotype III, V, and Ia. These results are notable because of the high prevalence of these serotypes in acute *S. agalactiae* UTI (16).

In summary, the growth experiments performed in this Chapter using human urine with 358 *S. agalactiae* isolates from three different patient groups allowed us to demonstrate that 1) certain *S. agalactiae* isolates can utilize human urine for growth 2) High-grade ABU *S. agalactiae* isolates exhibit the best fitness for growth in human urine when compared to acute UTI group 3) Serotyping of selected isolates demonstrated that 35% of fast growing isolates were serotype III. These results
indicate that individuals with high-grade ABU are significantly more likely to be colonized with a fast grower compared to patients with cystitis or low-grade ABU. This is the first demonstration of an association between different clinical conditions of S. agalactiae UTI and the bacteria’s ability to utilize human urine for growth. Thus, like other uropathogens such as E. coli, S. agalactiae also possesses specific traits that allow survival in the urinary tract and urine specifically. Further detailed studies on genome sequencing, molecular characterization and growth experiments in single carbon source or other individual metabolites which are available in human urine will pave way to the better understanding on the factors that contribute to uropathogenic S. agalactiae survival trait and its pathogenicity.
Chapter 5

Discovery and characterization of human urine utilization by asymptomatic bacteriuria Streptococcus agalactiae

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, is:


Infection and Immunity (Under revision).

My contribution to the paper involved:
Designing and optimisation of the experiments and generation of data for all growth experiments, serotyping, competition assays, generating mutants (knocking-out gene of interest), data analysis and writing of the manuscript.

(Signed) _________________________________ (Date)______________

Deepak S. Ipe

(Countersigned) ________________________________ (Date)______________

Corresponding author of paper: Glen Charles Ulett

(Signed) _________________________________ (Date)______________

Supervisor: Glen Charles Ulett
Introduction

*Streptococcus agalactiae* is a leading cause of infection in newborns, pregnant women, and older persons with chronic medical illness (12). This organism also causes urinary tract infections (UTI) including cystitis (16) and asymptomatic bacteriuria (ABU) (36, 43). These infections have been associated with diverse patient groups including pregnant and non-pregnant women, and elderly individuals (16, 17, 34, 41, 48, 50). Bacteriuria due to *S. agalactiae* in pregnant women is especially important because of the potential risk for vertical transmission of infection. *S. agalactiae* bacteriuria has an overall incidence of between 1.0-3.5% in individuals with suspected UTI (27, 36, 42, 54, 184). In one study, *S. agalactiae* was recovered from 26% of cases of ABU in diabetes gravidas (382). Bacteriuria of any count due to *S. agalactiae* in pregnancy is routinely treated with antibiotics to minimize the risk of vertical transmission to the neonate, as noted elsewhere (189, 190, 256, 389). In absolute cell numbers, *S. agalactiae* bacteriuria loads in infected individuals average between 50,000 and 70,000 CFU ml⁻¹ during UTI (43), but these counts are known to be dynamic and may change dramatically over a few hours (16). Additionally, the overall clinical significance of *S. agalactiae* bacteriuria in certain patient populations, and the cost benefit of universal treatment for this condition in pregnancy, remains unclear (183, 193). Finally, it is unknown whether *S. agalactiae* bacteriuria might act as a reservoir for persistence, or contribute to chronic ongoing infection in the host.

One of the important fitness traits that contribute to the persistence of bacteria such as ABU *Escherichia coli* in the lower urinary tract is the ability to grow in human urine. This enables bacteria such as *E. coli* to persist in the host by re-growth of non-voided organisms in residual urine, and offers a competitive advantage independent of urothelial cell binding and inflammation (261, 262). In addition to *E. coli* other
organisms have also been reported to grow in human urine including staphylococci (275) and enterococci (273). For this, bacteria must endure low pH, high urea levels, nutrient limitation, nitrite in mildly acidified urine, and exposure to antimicrobial proteins secreted in urine, such as Tamm-Horsfall protein. Several groups have studied the metabolic basis for urine growth of ABU E. coli, which has shown links to transport and degradation pathways for galacturonate, glucuronide and galactonate (261), as well as to synthesis of guanine, arginine, and glutamine (384). Antioxidant defense mechanisms have also been identified as being important for urine growth of E. coli (266). Russo et al., described that synthesis of guanine-dependent products was critical for this phenotype in urine (270). Arginine metabolism was shown to contribute to the growth of E. coli in another study (271). In addition, tolerance to high levels of, or requirement for, D-serine has been linked to the survival of Staphylococcus saprophyticus (275) and E. coli in urine (384), respectively. Finally, iron acquisition has been identified as potentially important in E. coli (278) and E. faecalis (273) for urine growth.

UPSA isolated from individuals with acute cystitis and pyelonephritis, were shown to be incapable of human urine growth in two prior studies (87, 88). While these organisms adhered to urothelial cells and induced inflammatory responses they had no capacity for urine growth; a phenotype similar to those previously documented in a prior study on S. agalactiae (263). The potential for growth of ABSA in human urine has not previously been investigated. In this study, we describe, for the first time, a novel fitness trait of robust growth of ABSA in human urine, which was not observed in multiple UPSA clinical strains. These findings establish that some S. agalactiae are capable of robust urine growth, which we show is related, in part, to malic acid metabolism.
Material and methods

Bacterial strains

The initial ABSA strain used in this study was cultured from urine obtained by catheter from an asymptomatic 26-year-old pregnant woman undergoing routine screening. Repeat urine cultures grew pure *S. agalactiae* at 50,000 CFU ml⁻¹, which continued over a five-week period and the woman was diagnosed with ABU. The strain was referred to as ABSA 1014. The UPSA isolate referred to as UPSA 807 was used for comparative studies with ABSA 1014. UPSA 807 was cultured from a clean-catch voided urine sample obtained from a 59-year-old woman who presented with frequency, urgency, hematuria, pyuria and bacteriuria of 80,000 CFU ml⁻¹. Urinalysis was consistent with a clinical diagnosis of acute uncomplicated cystitis. *S. agalactiae* was identified and serotyped using latex agglutination as previously described (16). Details of several other isolates used for subsequent study are provided in Table 5.1. This study had approval from the University of Alabama committee on human experimentation (X070619009), and the Griffith University human ethics committee (MSC/06/08/HREC), in accordance with the Helsinki Declaration.

Human urine growth assays and culture conditions

For growth assays, we used pooled urine collected from six healthy female and male volunteers who had no recent history of UTI or antibiotic usage (preceding month). Urine was combined in equal volumes, filter sterilized (0.45µM), stored at 4°C, and used within 48h. Growth of *S. agalactiae* in urine and Todd Hewitt broth (THB) was quantified using colony counts and turbidity measurements. Duplicate cultures were grown in 200µl volumes in 96-well microtiter plates inoculated with approximately 10³ CFU, which were prepared by washing (1XPBS, pH 7.4) and back-diluting 1/100,000 from an overnight culture. Cultures were grown at 37°C, under shaking conditions
(200rpm). Colony counts were performed using Todd Hewitt agar (THA), with antibiotic selection as indicated, and optical density (OD) at 600nm was measured to monitor culture turbidity. Growth assays were repeated three to five times in independent experiments using different batches of pooled urine. Based on results from initial growth assays, we also performed direct competition assays to determine whether ABSA 1014 could out-compete any of the UPSA isolates in urine. These assays were designed using differential susceptibilities of ABSA 1014 and UPSA isolates to clindamycin and tetracycline, respectively (Table 5.1). For competition assays, ABSA 1014 and one UPSA isolate were mixed at a 1:1 ratio and incubated in pooled human urine for 72h. The numbers of each strain were determined by plating onto selective THA (5µg ml⁻¹ clindamycin, or 20µg ml⁻¹ tetracycline; only permitting growth of UPSA strain) and nonselective THA (permitting growth of both ABSA and UPSA) that enabled calculation of the relative proportions of each strain over time.

**Comparative phenotype metabolic arrays**

Based on the initial urine growth data, we undertook a comprehensive comparison of the metabolic profiles of ABSA 1014 and UPSA 807 using Phenotype Metabolic (PM) array fingerprinting (Biolog Inc., Hayward, CA). These arrays enabled an analysis of the survival and metabolic activities of ABSA 1014 and UPSA 807 under nutrient limiting conditions that included >2500 substrates and physiologic exposures incorporating carbon, nitrogen and phosphorus utilization, biosynthetic pathway and nutrient stimulation, osmotic/ionic and pH responses, and chemical sensitivity. A complete list of the test conditions (integrating PM1-20 arrays) is available at http://www.biolog.com. Cultures in array plates were incubated at 37°C for 24-48h and the data were collected using an OmniLog V1.5 module. The pair-wise comparisons were performed using two independent experiments and biological duplicates in each.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Source(^a)</th>
<th>Presentation(^a)</th>
<th>Bacteriuria (^b)</th>
<th>Sero(^c)</th>
<th>ST(CC)(^d)</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSA 1014</td>
<td>26, F</td>
<td>Asymptomatic</td>
<td>50,000 cfu ml(^b)</td>
<td>II</td>
<td>28(19)</td>
<td>Gentamicin</td>
</tr>
<tr>
<td>ABSA 729</td>
<td>69, F</td>
<td>Asymptomatic</td>
<td>30,000 cfu ml(^b)</td>
<td>NT</td>
<td>452(24)</td>
<td>Gentamicin</td>
</tr>
<tr>
<td>ABSA 834</td>
<td>57, F</td>
<td>Asymptomatic</td>
<td>50,000 cfu ml(^b)</td>
<td>III</td>
<td>182(19)</td>
<td>Gentamicin</td>
</tr>
<tr>
<td>UPSA 807</td>
<td>59, F</td>
<td>Freq/Urgency, Gross Hemat, Pyuria</td>
<td>80,000 cfu ml(^1)</td>
<td>V</td>
<td>1(1)</td>
<td>Gentamicin, Clindamycin, Tetracycline</td>
</tr>
<tr>
<td>UPSA 714</td>
<td>89, F</td>
<td>Dysuria, Pyuria, Gross Hemat</td>
<td>50,000 cfu ml(^1)</td>
<td>III</td>
<td>17(17)</td>
<td>Gentamicin, Tetracycline</td>
</tr>
<tr>
<td>UPSA 058</td>
<td>77, M</td>
<td>Dysuria, Pyuria, Freq/Urgency, Gross Hemat</td>
<td>100,000 cfu ml(^1)</td>
<td>Ia</td>
<td>23(23)</td>
<td>Gentamicin, Tetracycline</td>
</tr>
<tr>
<td>UPSA 008</td>
<td>57, F</td>
<td>Dysuria, Gross Hemat</td>
<td>100,000 cfu ml(^1)</td>
<td>V</td>
<td>1(1)</td>
<td>Gentamicin, Clindamycin</td>
</tr>
<tr>
<td>UPSA 872</td>
<td>69, F</td>
<td>Dysuria, Pyuria, Gross Hemat</td>
<td>50,000 cfu ml(^1)</td>
<td>Ia</td>
<td>1(1)</td>
<td>Gentamicin, Tetracycline</td>
</tr>
</tbody>
</table>

\(^a\)Source, denotes age, gender of patients from whom S. agalactiae was cultured (F, female; M, male). Hemat, denotes hematuria; \(^b\)Detected at an equal or higher count in repeat urine samples; \(^c\)Sero, serotype; \(^d\)ST, sequence type and (CC), clonal complex
The final tabulated datasets represent all of the metabolic phenotype differences that were observed, and consistent, in comparisons of the two strains in independent array experiments.

**DNA isolation, genome sequencing and bioinformatic analysis**

To investigate the genetic basis for the metabolic differences we observed between ABSA 1014 and UPSA 807 we performed whole genome sequencing followed by comparative genomic analysis. Genomic DNA was isolated from overnight cultures of *S. agalactiae* grown in THB, centrifuged (8,000 × g, 10min), and re-suspended in 395μl DNA isolation buffer (50mM Tris-HCL, 0.145M NaCl, pH7.5) with 5μl mutanolysin (10U μl⁻¹). The cells were incubated for 90min at 37°C with shaking, followed by the addition of 154μl DNA isolation buffer, 30μl 10% SDS, 6μl proteinase K (20mg ml⁻¹) and 10μl RNase A (20mg ml⁻¹). Following 60min incubation at 37°C with shaking, 100μl of 5M NaCl was added and mixed. The remaining protocol for DNA isolation used the standard CTAB method (390). Genomic DNA from ABSA 1014, UPSA 807 and six other strains listed in Table 5.1 was used to generate 100bp paired-end reads using the Illumina HiSeq2000 platform. Reads were then assembled using velvet (391) and contigs ≥200 bp were annotated using prokka (http://www.vicbioinformatics.com/software.prokka.shtml). Genome comparisons of syntenic loci were performed using Artemis Comparison Tool (ACT) (392) and comparative genome images were generated using Easyfig (393). In silico antibiotic and virulence gene profiles were generated using BLAST (394) and SeqFindR (http://github.com/mscook/SeqFindR) using both assembled and mapping consensus modes to allow for read data to be used to identify query sequences that might not have assembled properly, such as repeat-containing sequences. The genomic context of *tetM*, *ermA*, and *ermB* were determined using ACT (392).
Targeted disruption of the malate metabolic pathway in ABSA

Based on the metabolic and genomic data derived from comparisons of ABSA and UPSA we next functionally inactivated the ABSA malate metabolic pathway for comparative assays in urine growth experiments. Deletion of the malic enzyme gene maeE in ABSA 834 was performed as described elsewhere (135) with modifications (ABSA 834 was chosen since ABSA 1014 was found to be not readily transformable in initial assays and ABSA 834 showed identical urine growth properties to ABSA 1014). A 595-bp fragment corresponding to the sequence 5’ end of the maeE coding sequence and a 537-bp fragment corresponding to the 3’ maeE terminus and flanking regions were amplified by PCR using the primer pairs: Mae-forCm-Up-F4, 5’-ACAACTCGAGCCATCAAACCTGGCACTGTAGG-3’ (XhoI), and Mae-forCm-Up-R4, 5’-CCAATTTCGTTTGGTAACTAAAGTTGCGGCAGCGAGGG-3’; and Mae-forCm-Down-F4, 5’-CTAATGTCAACTACCTGCCCGCGCATCAGTTCATTGGTCTG-3’, and Mae-forCm-Down-R4, 5’-AGAAGCGGCCGCAACCACACTTTGCTGGTCAGGGG-3’ (NotI), respectively based on the S. agalactiae 2603V/R genome. Underlined bases not matching the genes cds were incorporated into primers to produce restriction-endonuclease sites for ligation. The chloramphenicol acetyltransferase (cat) gene was amplified from pLZ12 (395) for cloning and selection using primers Cm-ampli-F, 5’-TTAGTTCAACAAACGAAAATGG-3’, and Cm-ampli-R, 5’-GGGGCGAGGTTAGTGACATTA-G-3’. The upstream reverse Mae-forCm-Up-R4 and downstream forward Mae-forCm-Down-F4 primers incorporated 5’ overhangs complementary to cat (in bold) to facilitate a three-way sewing PCR using re-amplification primers Mae-forCm-Up-F4 and Mae-forCm-Down-R4 primers and equal amounts of the three amplicons to yield a deletion of the maeE gene from 1902179bp to 1903308bp inclusive. Amplification reactions were performed in 50μl volumes containing approximately 50ng of DNA, 0.2mM dNTPs, 1.5-4.0mM MgCl₂, 200nmol of
each primer, and 1 unit of High Fidelity Phusion DNA polymerase (Thermo Scientific) in the manufacturer’s supplied HF Phusion buffer. An initial denaturation for 2min at 98°C was used, followed by 35 cycles each consisting of 10s at 95°C (denaturation), 50s at 65°C (annealing), and 30s/kb at 72°C (extension), with a final extension for 4min at 72°C. The mutant \textit{maeE} gene was gel-purified (QIAquick Gel Extraction kit, Qiagen) and cloned into the targeting vector pHY304-aad9 that was modified from pHY304 (117) by introducing the spectinomycin (Spt) resistance gene \textit{aad9} from pUCSpec (396) into the multiple cloning site for use in erythromycin resistant \textit{S. agalactiae} (16). For this, the PCR amplicon and pHY304-aad9 were digested with XhoI and NotI, and ligated to create pDI102, which was transformed into \textit{E. coli} DH5α. Transformants were screened on LB agar with Spt (100µg ml\(^{-1}\)) and chloramphenicol (Cm; 20µg ml\(^{-1}\)) and confirmed by restriction digest, PCR and sequencing. This strain, GU2136, was used to purify pDI102, which was electroporated into competent ABSA 834, as described (397, 398), to generate GU2293. An overnight culture of GU2293 grown in THY with Spt (100µg ml\(^{-1}\)) at 28°C was back-diluted 1:50, incubated for 2.5h at 28°C, then shifted to 37°C for 2.5h to induce integration of the targeting vector. The culture was then diluted 1:50 in fresh THY with Spt, and incubated overnight at 37°C. This culture was then again diluted 1:50 in fresh THY without antibiotics and incubated for 4h at 28°C, and then transferred to 37°C for 6h. Shaking at 200rpm was provided to all cultures. Ten-fold serial dilutions were performed in 1XPBS and plated onto THY agar with Cm (10µg ml\(^{-1}\)) that was incubated at 37°C. These colonies were patched and screened for a Spt\(^S\), Cm\(^R\) phenotype, representing a double crossover chromosomal integrant. Genomic DNA was prepared from a Spt\(^S\), Cm\(^R\) colony and analyzed using PCR and DNA sequencing to confirm the ABSA 834Δ\textit{maeE} mutant, GU2296.
Results

Growth of ABSA and UPSA in human urine

Initial examination of two different UPSA strains from patients with cystitis, and other S. agalactiae isolates from prior studies, showed that these organisms grew very poorly in human urine, a finding that was consistent with prior studies (87, 88, 263). Based on these data we tested and exposed a novel phenotype of efficient growth in human urine for ABSA 1014 that was not observed in several UPSA strains, including UPSA 807 (Fig 5.1A). The average generation time of ABSA 1014 was 170 min (calculated between 0-36h), compared to UPSA 807 and other UPSA strains that grew poorly with generation times >600 min (two-sample two-tailed t test, P<0.001). Subsequent assays of several other UPSA and ABSA strains, that were isolated from different patients with acute UTI and individuals with ABU respectively, showed similar urine growth patterns (Fig 5.1B). Thus, these data establish that multiple ABSA strains can grow robustly in human urine, in contrast to UPSA strains that grow poorly in urine.

The rapid doubling time of ABSA 1014 in human urine compared to UPSA indicated that this strain might out-compete UPSA strains in direct competition assays. In urine that was inoculated with equal numbers of ABSA 1014 and UPSA 807, or any one of several other UPSA strains (mixed 1:1 at start of assay), the percentage of each strain recovered over time showed the markedly superior fitness of ABSA 1014 for human urine growth compared to the UPSA strains (Fig 5.1C). In these assays, ABSA 1014 constituted 50% of the mixed population at t=0h, but >99% of mixed cultures after 24h (paired two-tailed t test, P<0.001). The superior growth phenotype of ABSA 1014 was urine-specific because all of the strains had equivalent growth in THB, as illustrated in Fig 5.1D. Thus, ABSA 1014 outcompetes multiple UPSA strains for rapid growth in
human urine through a urine-specific growth trait evident by an absence of any general growth defect in UPSA strains detectable using standard laboratory media.

**Metabolic profiling of ABSA 1014 and UPSA 807 using PM arrays**

We next undertook a comprehensive comparison of the core physiological properties of ABSA 1014 and UPSA 807 using PM arrays to gain insight into the metabolic potential of these strains. The arrays uncovered only a few metabolic differences between the strains; in fact, there were a total of only four gained phenotypes in ABSA 1014. These were: efficient utilization of D, L-Malic acid and L-Malic acid, and resistance to sodium arsenate and cadmium chloride. In addition, there were several phenotypes that were absent in ABSA 1014 compared to UPSA 807, including resistance to macrolides and lincomycin. A complete list of these metabolic profiles is provided in Table 5.2. These findings were noted since L-Malic acid is a common organic acid in many fruits and vegetables, is synthesized in kidney cells and is often present in human urine (http://www.hmdb.ca/metabolites/hmdb 00156) along with pyruvic acid (281) from which it can be derived. Its concentration in urine is dramatically affected by dietary intake (399). In bacteria, a metabolic signature of L-Malic acid utilization is related to functional expression of the malic enzyme (ME) metabolic pathway, as characterized in *Lactobacillus* (289) and *Enterococcus* (400). A gene encoding a putative malate oxidoreductase has been annotated in the *S. agalactiae* reference 2603V/IR genome, however, the ME pathway has not been characterized in *S. agalactiae* to date. Malic acid metabolism is typically associated with an operon comprising genes encoding a malate oxidoreductase enzyme (*maeE*), permease/transporter (*maeP*), transcriptional regulator (*maeR*), and/or an accessory membrane-anchored sensor kinase (*maeK*) (401). Bacterial MEs catalyze the oxidative decarboxylation of malate to pyruvate and CO₂ (400), which underpins malolactic fermentation. Whilst there is some evidence that
MEs contribute to energy generation and bacterial survival at low pH, a role for MEs in bacterial growth in urine has not previously been investigated. Therefore, we examined the ME pathway in ABSA 1014 and UPSA 807 using genome sequencing, in silico and functional analyses.

**Draft genome assembly and analysis**

Interrogation of the draft assembled genomes of ABSA 1014 and UPSA 807 highlighted the presence of the *mae* gene cluster in both strains (Fig 5.2). The *mae* gene clusters from ABSA 1014 and UPSA 807 shared identical structural organization with the homologous *mae* cluster from *Lactobacillus casei* BL23 (289). Notably, however, a frameshift mutation in a poly A sequence at the 5’ end of *maeK* of UPSA 807 was identified. We hypothesize that this mutation would lead to the production of a truncated non-functional protein (using an alternative downstream ATG start codon) that lacks the first 19 amino acids corresponding to part of the signal peptide of this secreted protein. The mutation in UPSA 807, highlighted in Fig 5.2, was confirmed by PCR (primers uppS-F [5’-GTGATTTCTG AACGTCCGCCAT-3’] and galE-R [5’-ATACCGTAGGCTTGATCAGC-3’]) and sequencing of the entire *mae* gene cluster from both ABSA 1014 and UPSA 807. Further sequence comparisons revealed the presence of homologous gene clusters in all complete genomes of *S. agalactiae* available in the databases. We observed equivalent structural organization in all strains but noted several other unique disruptions, in particular in all non-human associated strains (SA20-06, ILRI112, ILRI005, 2-22 and 09mas018883). The details of these disruptions, and their occurrence in the *S. agalactiae* strains available in the databases, are illustrated in Fig 5.3.
Table 5.2: Metabolic profile of ABSA 1014 compared to UPSA 807 derived from PM array, and genotype relating to phenotype (substrate or molecule), determined using draft genome assemblies

<table>
<thead>
<tr>
<th>Metabolic Phenotype</th>
<th>Activity Level</th>
<th>Substrate or Molecule</th>
<th>Phenotype Relating to Substrate or Molecule</th>
<th>Genotype Relating to Substrate or Molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon Utilization</td>
<td>114</td>
<td>D, L-Malic acid</td>
<td>Carbon source, carboxylic acid</td>
<td>maeK frameshift in UPSA 807</td>
</tr>
<tr>
<td>Carbon Utilization</td>
<td>83</td>
<td>L-Malic acid</td>
<td>Carbon source, carboxylic acid</td>
<td>maeK frameshift in UPSA 807</td>
</tr>
<tr>
<td>Resistance</td>
<td>99</td>
<td>Sodium arsenate</td>
<td>Toxic anion, phosphate analog</td>
<td>ICEsde3396 in ABSA 1014</td>
</tr>
<tr>
<td>Resistance</td>
<td>305</td>
<td>Cadmium chloride</td>
<td>Toxic cation</td>
<td>ICEsde3396 in ABSA 1014</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>-148</td>
<td>Poly-L-lysine</td>
<td>Membrane, detergent, cationic</td>
<td>ND^a</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>-207</td>
<td>Alexidine</td>
<td>Membrane, e^- transport, biguanide</td>
<td>ND^a</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>-253</td>
<td>Lincomycin</td>
<td>Protein synthesis, lincosamide</td>
<td>Tn6002 in UPSA 807</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>-550</td>
<td>Oleandomycin</td>
<td>Protein synthesis, macrolide</td>
<td>Tn6002 in UPSA 807</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>-465</td>
<td>Troleandomycin</td>
<td>Protein synthesis, macrolide</td>
<td>Tn6002 in UPSA 807</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>-207</td>
<td>Spiramycin</td>
<td>Protein synthesis, macrolide</td>
<td>Tn6002 in UPSA 807</td>
</tr>
</tbody>
</table>

^aND, not determined
Fig 5.1: Growth of ABSA and UPSA Isolates and Direct Competition Assays

Fig 5.1: (A) Robust growth of ABSA 1014 in urine contrasts to poor growth of UPSA strains 807, 714 and 058. (B) High growth phenotypes of additional ABSA strains 729 and 834 in human urine compared to UPSA strains 008 and 872. (C) Direct competition assays revealed markedly superior fitness of ABSA 1014 for growth in urine versus UPSA 807, 714, 008 and 872; *OD600nm of 1014-807 co-culture shown; similar to other cocultures. (D) Robust growth of all strains in THB showed no general growth defect of UPSA strains.
Fig 5.2: Organization of the *mae* Locus in ABSA 1014 and UPSA 807. *maeK* is a pseudogene in UPSA 807 due to a single deletion occurring in an homopolymeric tract at position +12 in *maeK*. A potential in-frame alternative start codon can be found downstream at position +52.

![Diagram showing the organization of the mae Locus in ABSA 1014 and UPSA 807.](image)

<table>
<thead>
<tr>
<th></th>
<th>maee</th>
<th>maep</th>
<th>maeK</th>
<th>maeR</th>
<th>Accession</th>
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<td></td>
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<td>ERS086595*</td>
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<td>2-22</td>
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<td>09mas018883</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Legend:**
- **gene is intact**
- **gene is truncated**
- **gene has 3 frameshifts**
- **gene has 1 frameshift mutation at N terminus, -19 amino acids (AA)**
- **1 frameshift at C terminus -3 AA but mutation unlikely to disrupt function**

Fig 5.3: Mutations in *mae* Genes in ABSA and UPSA Strains in this study *(shown with accession numbers, Bioproject ID: PRJEB2837)*, and in the complete genomes of *S. agalactiae* strains available in GenBank.
High levels of antibiotic resistance, in particular to erythromycin, clindamycin and tetracycline have been described among *S. agalactiae* UTI isolates (16); thus, we sought to also define the genetic basis of antibiotic resistance in ABSA 1014 and UPSA 807. In UPSA 807, resistance to tetracycline and clindamycin could be explained by the presence of the conjugative transposon Tn6002 encoding the *tetM* and *ermB* genes, while in ABSA 1014, tetracycline resistance is related to the presence of the *tetM*-containing conjugative transposon Tn916. A summary of these findings, along with those for several other potential virulence genes, are illustrated in Fig 5.4. Resistance to cadmium and arsenate in ABSA 1014 is associated with the integrative conjugative element ICESde3396 (Table 5.2). In terms of the prevalence of genes encoding potential virulence factors that we hypothesize could have relevance for infection we examined the major toxin, antigen and protease genes in ABSA 1014, UPSA 807, and six other strains for which sequencing was performed. This analysis revealed that the genes for several factors including the beta-hemolysin/cytolysin, acid phosphatase *AcpC*, CAMP factor *Cfb*, and the surface immunogenic protein *Sip* were present among all strains tested. In contrast, the genes for other factors including the alpha C protein antigen, the protease immunity protein *Rib*, and the C5a peptidase *ScpB* were found irregularly in ABSA and UPSA strains (Fig 5.4). Thus, among the strains tested, we found no discernable pattern of virulence genes that would discriminate ABSA from UPSA.

**Growth of ABSA and a malate pathway-deficient ABSA mutant in urine**

We next sought to determine whether a functional ME metabolic pathway in ABSA contributes to the robust urine growth phenotype that we observed in these strains. For this, we inactivated the ME pathway by generating a *maeE*-deficient mutant in ABSA 834, since ABSA 1014 was not easily transformable in initial assays, and we observed that ABSA 834 exhibited identical urine growth properties to ABSA 1014. The results of the growth assays comparing a ME-deficient ABSA 834Δ*maeE* mutant to the wild-type (WT) ABSA 834 strain in pooled
normal human urine (NHU) and synthetic human urine (SHU), both containing 40mM malic acid, are shown in Fig 5.5. We used SHU in these assays for reproducibility between independent experiments and to enable direct comparison with prior urine bacterial growth studies. The SHU used was based on prior studies (354, 364). In these assays, the ABSA 834ΔmaeE mutant was significantly attenuated for growth in both NHU and SHU compared to the WT ABSA 834 strain; this phenotypic difference between the strains was most apparent after 24h. Importantly, both the mutant and WT strain had virtually identical growth curves in THB over the time course (data not shown). Quantitative determination of the amount of malic acid that was present in the growth media at start of assays (t=0h) and at 72h using a commercial kit (Megazyme International, Bray, Ireland) showed that there was a 65-70% reduction in malic acid concentration after 72h following growth of the WT ABSA 834, but there was no reduction in the malic acid concentration in ABSA 834ΔmaeE cultures. Thus, an intact ME metabolic pathway is essential for malic acid catabolism in WT ABSA 834 and is required for optimal robust growth of ABSA in both NHU and SHU containing malic acid.
Fig 5.4: Antibiotic Resistance Genotypes and Other Virulence Genes Profiles in ABSA and UPSA Strains  Serotype, sequence type (ST) and clonal complexes (CC) are indicated for comparison purposes. The tetracycline resistance gene tetM and erythromycin resistance genes ermA and ermB were identified in several strains and were associated with various transposons as depicted in the legend. The virulence gene profile for toxins (acid phosphatase [acpC], CAMP factor [cfb], cytolysin [cyl operon]), antigens (surface immunogenic protein [sip], alpha C protein antigen [bca], β-C protein [cba], alpha-like protein [alp2], protease immunity protein [rib]), and protease C5a peptidase [scpB] are shown; gene presence is indicated by a black rectangle.
Fig 5.5: Growth of WT and ME Pathway-Inactivated ABSA 834 in Pooled Normal Human Urine (NHU) or Synthetic Human Urine (SHU) Both Containing 40mM Malic Acid Growth of the WT was significantly higher than that of its malic enzyme-deficient ABSA 834::ΔmaeE mutant (area under the curve analysis, 0h-36h, P<0.01). Both of the strains grew normally in THB with identical growth curves over the same time course (data not shown). The graph represents one experiment representative of three independent repeat assays with n=1.
Discussion

The principle aim of this study was to determine whether ABSA might be capable of urine growth, which is a trait of some ABU E. coli (261, 262, 278), but has not been described in any previous studies on S. agalactiae. The central novel findings of this study are (i) the demonstration that ABSA can grow vigorously in normal human urine, in contrast to UPSA strains tested to date that grow poorly, (ii) the metabolome finding that shows the ability of ABSA 1014 to utilize malic acid in contrast to UPSA 807, (iii) the discovery that malic acid utilization in ABSA appears to correlate with an intact mea locus, and (iv) that, in ABSA, a functionally intact ME metabolic pathway appears to be required for optimal robust growth in human urine and affords activity for malic acid catabolism that takes place during urine growth. Complementation studies to restore functions of maeK and maeE will be important to confirm the findings in (iii) and (iv). More broadly, the literature on host-microbe interactions relating to ABU and the urinary tract shows that ABU E. coli minimizes urothelial cell binding to perhaps aid ‘immune-escape’ by minimizing activation of immune defenses. This may promote persistence of ABU (261, 262, 278). In contrast, uropathogenic bacteria adhere to urothelial cells and induce inflammation (88, 141, 315). Prior studies showed that UPSA can adhere to urothelial cells at similar efficiency compared to uropathogenic E. coli; however, UPSA grew very poorly in urine (87, 88); a phenotype consistent with a study by Stamey and Mihara (263). In this study, the finding that ABSA can utilize urine for robust growth sheds new light onto how this organism may interact with the host environment in the urinary tract. This fitness trait could influence the persistence of S. agalactiae ABU in some individuals. This hypothesis, and the binding ability of ABSA strains to urothelial cells, will be areas to test using experimental models of UTI in future studies.

In defining the phenotypic ABSA metabolome, and comparing this with genetic and mutational analyses, this study provides key insight into the mechanisms underlying urine
growth for *S. agalactiae*. The inability of UPSA 807 to grow in urine correlated with a single metabolic deficiency of malic acid catabolism, as revealed using PM arrays. Genome-sequence comparisons with ABSA 1014 highlighted the predicted non-functional ME pathway in UPSA 807. Crucial point mutations predicted to impact the functional integrity of the ME pathway in *S. agalactiae* were confirmed in multiple UPSA strains by comparative genomics. In addition, the targeted generation of a maeE-deficient ABSA mutant showed that a functionally active ME pathway contributes significantly to the urine growth phenotype in ABSA and inactivation of this pathway restricts growth in an otherwise ‘robust’ strain in NHU and SHU containing malic acid. To our knowledge, this is the first description of a role for a bacterial ME metabolic pathway in human urine utilization. Interestingly, the presence of functionally intact ME pathways in two UPSA strains that grew poorly in urine (UPSA 714, 058) implies that ME divergence is not the sole metabolic distinction between all ABSA and UPSA strains underlying differential urine growth. Future studies using transcriptional profiling will now be of interest to explore the full gamut of gene activation pathways at play during ABSA growth in urine beyond malic acid metabolism.

Several bacterial species have the ability to metabolize malic acid through different biochemical pathways. In bacteria such as *E. coli* and *Bacillus subtilis*, malate utilization is controlled by a two-component system (402, 403). In lactic acid bacteria including *L. casei* (289) and *Streptococcus mutans* (404, 405) L-malic acid is converted to L-lactate using malolactic enzyme. Organisms such as *Enterococcus faecalis* (400, 406), *Streptococcus bovis* (407), and *L. casei* (289) metabolize L-malic acid to pyruvate using the ME pathway. Oral streptococci degrade malic acid to create an alkaline environment, which may protect the bacteria from acid damage, oxidative and starvation stress (404, 405, 408). Preferential utilization of malic acid via the ME pathway may therefore enable an organism to survive and grow more efficiently at a lower pH (289). In our study, growth of ABSA in the acidic and low pH environment of human urine (pH 5.8-6.2), depended on a functional ME pathway. Future
investigations will now be needed to address precisely the exact substrate level phosphorylation and energy production pathway involved in this process and the involvement of pyruvate and/or lactate.

Prior studies have shown that uropathogenic bacteria including *E. coli*, *E. faecalis* and other enterococci can utilize human urine for growth and survival (263, 273, 384). Urinary components including D-serine, guanine, and iron have been shown to influence the growth and/or survival of these organisms in this environment. However, urinary components that *S. agalactiae* uses to grow in urine have not previously been identified. In our study, we demonstrated that ABSA can metabolize the naturally excreted form of L-malic acid that is available in human urine (281, 399, 409). The amount of L-malic acid present in urine varies dramatically depending on dietary intake. L-Malic acid is a widespread organic acid found at especially high levels in currants, rhubarb, green apples, grape musts (404, 410), pumpkin fruits (411) and wine. In terms of other variable components in human urine, the presence of glucose (281) or fructose may inhibit certain metabolic pathways including ME pathway activity in bacteria including *S. lactis* (412), *E. faecalis* (406) and *L. casei* (289). Considering the variability of these components including L-malic acid levels in human urine, we used a standardized SHU media to compare the growth of WT and mutant ME-deficient ABSA using consistent experimental conditions. Synthetic media to mimic human urine has been used in several studies on the growth of bacteria to standardize experimental conditions, although the precise components that bacteria utilize in SHU for growth have not been specifically defined in most cases. The activity of the ME pathway also depends on divalent co-factors such as Mn$^{2+}$ or Mg$^{2+}$ (413) but the requirement for these co-factors in ABSA malic acid metabolism in human urine remains to properly defined.

Genome analysis and comparison of ABSA and UPSA showed similar virulence gene profiles for toxins including acid phosphatase (*acpC*), CAMP factor (*cfb*), cytolysin (*cyl operon*)
and the surface immunogenic protein (sip). The gene encoding alpha C protein (bca) was only present in one of the three ABSA strains, and was not found in any of the five UPSA strains tested in this study. Previous studies showed that S. agalactiae strains that express alpha antigen were less readily killed in the absence of antigen specific antibody than alpha negative strains (414). Surprisingly, no ABSA or any UPSA strains showed the presence of the genes encoding β-C protein (cba) or alpha-like protein (alp2). The protease C5a peptidase gene (scpB) was absent in UPSA 807 and UPSA 008 only. The absence of scpB might lead to less binding of human complement component C5a (90, 415, 416) although future studies will be needed to test this hypothesis.

The findings of the current study can also be compared more broadly to the literature in terms of implications for pathogenesis and infection in UTI. Recent data from Kline et al., showed that presence of S. agalactiae in the urinary tract in mice can predispose the host to other uropathogenic bacteria such as E. coli (326). Our findings suggest that ABSA strains that colonize urine more efficiently may have the potential to modify the progression of UTI due to other causal organisms in some individuals. Furthermore, S. agalactiae has the ability to modulate host immune responses in the bladder and studies to define potential differences between ABSA and UPSA in terms of immune modulation will now be important. The discovery of a novel phenotype of robust growth in human urine by ABSA, and the ability of ABSA to utilize malic acid in urine has implications for our understanding of the mechanisms used by this organism to maintain infection in the form of ABU, and will now provide the basis for further studies.
Chapter 6

Comparison of bladder colonization potential and urine growth of asymptomatic bacteriuria and uropathogenic *Streptococcus agalactiae* in murine model

My contribution to this Chapter involved:
Design of experiment and generation of data for all growth and mouse experiments, clinical description of isolates, analysis of the experimental results, and writing of the Chapter.

(Signed) _______________________________ (Date)______________
Deepak S. Ipe

(Countersigned) _______________________________ (Date)______________
Supervisor: Glen Charles Ulett
Introduction

Urinary Tract Infection

Urinary tract infections (UTI) are one of the most frequent infectious diseases in humans. Women are more susceptible to UTI than men and it has been reported that 1 in 3 women will have had at least 1 episode of UTI requiring antibiotic treatment by the age of twenty four (417-419). UTI contribute over 7 million hospital visits and admission per year with a significant economic burden to health care systems in the United States (326, 417). Gram positive uropathogens such as S. agalactiae can cause acute UTI, but have not been investigated intensively. This is in contrast to a great deal of knowledge from years of investigation on the most frequent UTI causative organism, E. coli that contributes up to 80% of cases (420).

At the broadest level, S. agalactiae UTI can be mainly classified into acute or symptomatic and asymptomatic bacteriuria (ABU) urinary tract infections. However, the full spectrum of S. agalactiae UTI includes cystitis, pyelonephritis, urosepsis and ABU. A recent case report described an unusual disease etiology in the form of acute S. agalactiae cystitis and massive abdominopelvic abscess, which was secondary to the S. agalactiae UTI (37). These infections have been reported as particularly common among some groups of diabetic patients, pregnant women, elderly and immunocompromised individuals with ABU accounting for many infections (12, 16, 32, 34, 36, 49). Diagnosis of ABU includes the presence of ≥10^5 CFU/ml bacteria in urine culture without any symptoms (421). This is in contrast to diagnosis of acute UTI, which includes the presence of ≥10^5 CFU/ml of uropathogens in urine with any one symptom such as fever (>38°C), urgency, frequency, dysuria, and suprapubic pain. Diabetes mellitus is linked to UTI as a risk factor because patients with diabetes mellitus are more likely to experience UTIs (422-424). For example, women with type 2 diabetes mellitus and ABU are more likely to develop acute UTI than women without ABU (172, 425). S. agalactiae UTI has
become more common in pregnant women and women with diabetes mellitus, and can lead to complications such as pyelonephritis and chorioamnionitis (257).

Colonization of the urinary tract or bladder with bacteria is the first step in causing UTI (141, 261). Bacteria possess a number of virulence factors to aid colonisation and cause UTI, and overcome innate responses from the host. For example, in UPEC, the mannose-binding type 1 pilus tip adhesin FimH helps the bacteria attach to and invade bladder epithelial cells, promoting colonisation (326). This is an important step in the pathogenesis of UPEC UTI. *S. agalactiae* has been shown to colonize mouse bladder *in vivo*, and human bladder uroepithelial cells *in vitro* (5637 and T24 cell lines) (87, 103, 138, 326). In addition, quantitative measures of binding of UPSA to human uroepithelial cells revealed that UPSA bound in statistically significantly higher numbers (*P* < 0.001) compared to a non-UPSA strain (87).

The ability of bacteria to grow in urine is another aspect that can influence bladder colonisation and UTI (256). In the prior Chapter 5, it was shown that malic acid utilisation may have a role in optimal growth of ABU *S. agalactiae* in human urine. Virulence factors in *S. agalactiae* related to UTI and bladder colonisation have not been well described. However, more generally, *S. agalactiae* virulence factors mainly include pore-forming toxins (β-hemolysin and Christie Atkins Munch Peterson ‘CAMP’ factor), and the sialic acid-rich capsular polysaccharide (CPS) (100, 115, 426). *S. agalactiae* CPS is rich in sialic acid (a nine carbon sugar, which is also present on the surface of glycoproteins) and appears to inhibit activation of host immune responses (427, 428). CPS also helps to prevent complement factor C3 deposition and *S. agalactiae* phagocytosis by the host immune system (426, 429). Other *S. agalactiae* virulence factors involved in host immune evasion are superoxide dismutase (SodA), serine protease C5a peptidase (ScpB), fibrinogen binding protein (FbsA), laminin binding protein (Lmb), serine rich repeat (Srr-1) protein, immunogenic bacterial adhesin (BibA) and surface anchored alpha C protein (ACP) (426, 430-437).
Bacteria infecting the urinary tract trigger innate immune responses, which are aimed at clearing the infection and which the bacteria must overcome to survive and persist in the host. These innate immune responses involve the induction of cytokines and chemokines. For \textit{S. agalactiae} UTI, induction of interleukin 1alpha (IL-1\(\alpha\)) has been reported in response to bladder colonization (87). In addition, one study reported high expression of CXCL-10 for the bladder response to \textit{S. agalactiae}. The study also showed uroepithelial cells defend against \textit{S. agalactiae} through the production of several cytokines and chemokines including IL-1\(\beta\), IL-1ra and IL-8 at 24h post infection (88). \textit{S. agalactiae} has also been shown to suppress or modulate host immune response. For example, a \textit{S. agalactiae} strain expressing minimal capsule sialic acid/O-acetylation (OAc\(\alpha\)) suppressed lipopolysaccharide (LPS) induced NF-kB driven inflammation (326). Kline \textit{et al.}, previously reported that acute \textit{S. agalactiae} infection produced several proinflammatory cytokines including IL-1\(\alpha\), macrophage inflammatory protein-1\(\alpha\) (MIP-1\(\alpha\)), MIP-1\(\beta\), IL-9 and IL-10. IL-10 is commonly considered as a cytokine able to control the degree and duration of an inflammatory response (103). However, the role of different cytokines and chemokines in controlling \textit{S. agalactiae} infection remains unknown. It has not been investigated whether there are any differences in cytokine and chemokine responses following challenge with UPSA as opposed to ABSA.

Prior chapters established the fact that ABSA isolates can grow better in human urine when compared to UPSA isolates. ABSA can also out-compete UPSA in human urine when grown together in a 1:1 ratio (Chapter 5). This chapter aims to (i) compare the ability of ABSA and UPSA to establish bladder colonization in the frequently used mouse model based on female C57BL/6 mice, (ii) investigate the effect of ABSA \textit{maeE} (encoding the malate oxidoreductase used for malic acid metabolism) on mouse colonization and (iii) compare the differential immune or inflammatory response of the mouse bladder to ABSA and UPSA.
Materials and methods

Streptococcus agalactiae Isolates

All *S. agalactiae* isolates used in this study were obtained from University of Alabama at Birmingham Hospital between August 2007 and August 2008. Isolates used in this study were grouped into two groups based on the UTI condition; (1) ABSA; cultured from ABU patients with \(>10^4\) CFU/ml and (2) UPSA; cultured from symptomatic patients who presented with acute UTI, and \(>10^4\) CFU/ml. The ABSA isolates used in this study were ABSA 834 and ABSA 1014. ABSA 834 was cultured from urine of an asymptomatic 57-year-old woman undergoing routine screening. Repeated urine cultures grew pure *S. agalactiae* at 50,000 CFU ml\(^{-1}\). ABSA 1014 was cultured from urine obtained by catheter from an asymptomatic 26-year-old pregnant woman undergoing routine screening. Urine cultures recovered pure *S. agalactiae* at 50,000 CFU ml\(^{-1}\), which continued over a five-week period. In both cases, women were diagnosed with high-grade \(>10^4\) CFU/ml) ABU. The UPSA isolate used in this study was UPSA 807. UPSA 807 was cultured from a clean-catch voided urine sample obtained from a 59-year-old woman who presented with frequency, urgency, hematuria, pyuria and bacteriuria of 80,000 CFU ml\(^{-1}\). Urinalysis was consistent with a clinical diagnosis of acute uncomplicated cystitis. Clinical history of all other isolates obtained during August 2007 and August 2008 is also detailed in the previous study (16). For some assays a mutant deficient in the *maeE* gene derived from WT ABSA 834 was used for comparisons. The mutant was generated and grown as described in the prior Chapter 5.

Preparation of bacterial inoculum

Frozen -80°C stocks of isolates were streaked on to Todd-Hewitt agar (THA) containing 5% defibrinated horse blood and incubated overnight at 37°C. Routine sub-cultures were prepared using THA containing 5% defibrinated horse blood. Broth cultures were grown in Todd-Hewitt broth (THB) overnight (~16h) at 37°C. Bacterial cells were then washed three times in cold 1XPBS and centrifuged for 10min (each wash) at 8000g. Cells were finally
resuspened in 1ml of cold 1XPBS (neat bacterial inoculum) and transferred to sterile 1.5ml Eppendorf tubes. A 1/100 dilution was performed to prepare the bacterial inoculum into a 1ml spectrophotometer cuvette (SARSTEDT) by adding 990µl of 1XPBS and 10µl of bacterial inoculum, and the optical density at 600nm (OD600nm) was measured. Colony counts were also performed from the bacterial inoculum retrospectively to provide an accurate viable cell count in CFU ml⁻¹, and correspond this with the OD600nm readings.

**Mouse challenge and tissue collection**

Bladder colonization experiments were performed using female C57BL/6 mice (8-12 weeks old; Animal Resources Centre, WA) to compare the ability of different ABSA and UPSA WT strains to infect mice, and the ability of WT ABSA 834 and ABSA 834ΔmaeE to colonize mice. Take inoculum in eppendorf tubes (1ml) to animal facility and gown-up with double gloves, booties, hair net, face mask. Mouse experiments were performed essentially as previously described (261). Briefly, mice housed in individual cages were lightly anaesthetized in a ~4% atmosphere of isoflurane. One-millilitre syringes full of inoculum and with a catheter attached to the syringe were used to infect the mice with a load of 40µl of bacterial suspension containing approximately 1X10⁸ CFU per dose. Control mice received 40µl of 1XPBS only. Prior to infection, the periurethral area was sterilized by swabbing with 10% povidone-iodine solution on a cotton swab for 5sec, which was removed with PBS on a cotton swab. Mice were catheterized under direct light by inserting the catheter directly into the bladder through the urethra. The catheter was removed following infection, and the mice returned to their cages for recovery. Group sizes of 10 were used for most experiments, and these were repeated at least twice. Ethics approval for animal work was provided under the approval number MSC/03/12/AEC from the Griffith University Animal Ethics Committee.

At the designated time point post-infection (typically 24h), urine and bladders were aseptically collected for colony counts and for immune response analysis. To collect urine, mice were picked up and held mouse over a small piece of parafilm to collect urine using gentle
abdominal massage and dispensed into a sterile 1.5ml eppendorf tube. Mice were then euthanized by cervical dislocation and bladder collected into 2.0ml CapLock Eppendorf Tube containing 120µl of 1X Protease Inhibitor (Roche diagnostics, Mannheim, Germany) in 1XPBS (one tablet per 10ml) and two stainless steel beads (5mm; Qiagen). Tissues were weighed and sample weights recorded.

Tissues were homogenized into single cell suspensions using a TissueLyzer (Qiagen), with two bursts of 1min each, at a setting level of 26 on the machine. After homogenisation, samples were centrifuged at 10,000 x g for 5min at 4°C, and two aliquots of supernatant of 50µl each were removed and dispensed into 96-well microtiter plates for storage at -80°C for subsequent immune responses analysis. The pellet was then resuspended with 920µl of 1XPBS and remix with the TissueLyzer for 30sec for colony counts using serial 10-fold dilutions across a microtiter plate down to 1/10,000 and 5µl of samples from each dilution plated in triplicate onto THA containing 5% horse blood. THA supplemented with 10µg/ml chloramphenicol antibiotics were used when appropriate. Data are expressed as CFU per ml or CFU per 0.1g of tissue with means and individual data points illustrated on the graphs.

**Immune response analysis**

Supernatants from two independent experiments were analysed at 24h post-infection using a 23-target multiplex protein assay (Bio-Rad Laboratories, Australia) as per the manufacturer’s instructions to assess production of cytokines and chemokines in response to ABSA and UPSA. The cytokines and chemokines tested, and their major functions and cellular sources, are listed in Table 6.1. Data are presented as concentration of protein per ml with each data point on the graph representing one single mouse, and the median group values also shown. Supernatants from all non-infected mice were also included from both independent experiments to determine the base level or normal level of cytokines and chemokines and were labelled as control (Ctrl).
<table>
<thead>
<tr>
<th>Name</th>
<th>Major Functions</th>
<th>Major Sources</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>Released in response to cell damage and can induce apoptosis</td>
<td>Monocytes and macrophages</td>
<td>(438-440)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Induces gene expression, synthesis of cyclooxygenase type 2 (COX-2), type 2 phospholipase A, inducible nitric oxide synthase (iNOS), prostaglandin-E2 (PGE2), platelet activating factor, and nitric oxide (NO), and mediates auto-inflammatory diseases</td>
<td>Monocytes, macrophage, dendritic cells (DCs), neutrophils, B cells and T cells, endothelial and epithelial cells</td>
<td>(439-441)</td>
</tr>
<tr>
<td>IL-2</td>
<td>Effects towards cytotoxic T cells, promotes cell survival, immune tolerance and apoptosis</td>
<td>CD4+, CD8+ T cells, dendritic cells (DCs), natural killer (NK) cells, and NKT cells</td>
<td>(442-446)</td>
</tr>
<tr>
<td>IL-3</td>
<td>Helps in growth and differentiation of CD34+ progenitor cells into hematopoietic cells like basophils and mast cells, myeloid-derived dendritic cells</td>
<td>T cells, macrophages and stromal cells</td>
<td>(447, 448)</td>
</tr>
<tr>
<td>IL-4</td>
<td>T helper (Th) type 2 cell activation, B cell activation and immunoglobulin (Ig) E secretion, mast cell development and maintenance of tissue and repair</td>
<td>Basophil, T helper type 2 cells (Th2), T follicular helper cells</td>
<td>(449-452)</td>
</tr>
<tr>
<td>IL-5</td>
<td>Differentiation, activation and survival of eosinophils</td>
<td>Th2 cells, eosinophils and mast cells</td>
<td>(448, 452, 453)</td>
</tr>
<tr>
<td>IL-6</td>
<td>B cell maturation, macrophage differentiation, induction of hepatic acute-phase proteins, promotes Th2 differentiation, tissue regeneration and homeostatic regulation</td>
<td>Monocytes, macrophages, DCs, fibroblasts, endothelial cells and T and B cells</td>
<td>(452, 454-456)</td>
</tr>
<tr>
<td>IL-9</td>
<td>Helps in maturation of hematopoietic</td>
<td>CD4+ T cells,</td>
<td>(457, 458)</td>
</tr>
<tr>
<td><strong>IL-10</strong></td>
<td>Regulates innate immune responses, facilitates the tissue healing process, can repress pro-inflammatory responses and limit excessive tissue damage</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IL-12p40</strong></td>
<td>Cell mediated immunity, induces differentiation of naive T cells into Th1 cells and Th1 mediated responses to disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IL-12p70</strong></td>
<td>Cell mediated immunity, induces differentiation of naive T cells into Th1 cells and Th1 mediated responses to disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IL-13</strong></td>
<td>Maintenance of tissue and repair</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IL-17A</strong></td>
<td>Promotes inflammatory immune responses, recruits neutrophils, enhances antibody production, and activates T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Eotaxin</strong></td>
<td>Recruitment of eosinophils and basophils from peripheral tissues and bone marrow</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>G-CSF</strong></td>
<td>Growth factor for neutrophils, helps in proliferation and differentiation of normal haemopoietic stem cells,</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GM-CSF</strong></td>
<td>Stimulates the proliferation of myeloid cells, DCs, myelomonocyte progenitors and responsible for protective immunity</td>
<td>T cells, B cells, DCs, macrophages, endothelial cells, fibroblasts and tumour cells (448, 478-480)</td>
<td></td>
</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td>Establishes cellular immunity, regulates immune function and connects the adaptive and innate immune response</td>
<td>Activated T lymphocytes and natural killer cells, monocytes, macrophage, B cells and DCs (452, 481-484)</td>
<td></td>
</tr>
<tr>
<td><strong>KC</strong></td>
<td>Effects on neutrophil trafficking</td>
<td>Macrophages and neutrophils (450, 485)</td>
<td></td>
</tr>
<tr>
<td><strong>MCP-1</strong></td>
<td>Effects on inflammatory monocyte trafficking</td>
<td>Macrophages, neutrophils and mast cells (450, 485, 486)</td>
<td></td>
</tr>
<tr>
<td><strong>MIP-1α</strong></td>
<td>Effects macrophage and natural killer cell migration, and T cell and DCs interactions</td>
<td>Macrophages, neutrophils and mast cells (450, 485, 486)</td>
<td></td>
</tr>
<tr>
<td><strong>MIP-1β</strong></td>
<td>Macrophage and natural killer cells migration, T cell and DCs interactions</td>
<td>Macrophages, neutrophils and mast cells (450, 485, 486)</td>
<td></td>
</tr>
<tr>
<td><strong>RANTES</strong></td>
<td>Macrophage and natural killer cells migration. T cell and DCs interactions</td>
<td>Macrophages, neutrophils and mast cells (450, 485-487)</td>
<td></td>
</tr>
<tr>
<td><strong>TNF-α</strong></td>
<td>Required for the formation of secondary lymphoid structures and mononuclear phagocytes,</td>
<td>(484, 488, 489)</td>
<td></td>
</tr>
<tr>
<td>antibody production</td>
<td>macrophages and DCs</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Statistics

Mean bacterial titres from bladders were compared using an independent sample t test with log-transformed data. Normal distribution parameters for the different data sets were estimated using P-P plots and histograms. P values <0.05 were considered significant. Welch's Independent t test was used to analyse mean cytokine levels for multiplex protein data with significance level set to p <0.05. Mean cytokine levels (pg/mL) that were not normally distributed were compared using a Mann–Whitney U test. Statistical analyses were carried out using IBM SPSS Statistics software (Version 20.0), and GraphPad Prism software package 5.0. Statistical significance was accepted as p <0.05.
Results

Bladder colonization and bacterial loads in urine

Bladder colonisation data for ABSA 1014 and UPSA 807, shown in Figure 6.1, revealed that there was significantly higher number of bacteria recovered from mice infected with ABSA 1014 compared to UPSA 807 at 24h post infection (p=0.02, Independent Samples t-test). In comparison, the numbers of bacteria in urine for ABSA 1014 were also significantly higher at 6h (p<0.031), and 24h (p<0.001) (but not at 12h) compared to UPSA 807 (Fig 6.2a-6.2c).

![Fig 6.1: Bladder Colonization](image)

Fig 6.1: Bladder Colonization of ABSA 1014 and UPSA 807 Data shows average bacterial CFU per ml in 100mg of bladder of ten mice per group at 24h post infection. Error bars are standard error of the mean (SEM). Data is representative of two independent experiments. Data were analysed using Microsoft Excel v14.0.7140.5002.
Fig 6.2a: Bacterial Loads in Urine

Fig 6.2b: Bacterial Loads in Urine

Fig 6.2c: Bacterial Loads in Urine

Fig 6.2a-6.2c: Bacterial Loads in Urine Shows the presence of bacteria (ABSA 1014 and UPSA 807) in mouse urine at 6h, 12h and 24h post infection in CFU/ml. Average values are calculated from ten mice (per group). Data is representative of two independent experiments. Error bars are calculated as SEM. Data were analysed using Microsoft Excel v14.0.7140.5002.
The numbers of \textit{S. agalactiae} CFU recovered from bladders of mice infected with WT ABSA 834 were significantly higher than mice infected with the ABSA 834\textit{maeE} mutant ($p=0.042$, Independent Samples t-test), as shown in Figure 6.3. In comparison, colony counts of urine collected from these mice at 6h, 12h and 24h post-infection were not different between the ABSA 834 and ABSA 834\textit{maeE} mutant strains (Fig 6.4a, 6.4b and 6.4c).

![Bladder Colonization](image)

\textbf{Fig 6.3: Bladder Colonization of ABSA 834 and ABSA 834\textit{maeE} mutant} Data shows average bacterial CFU per ml in 100mg of bladder of ten mice per group at 24h post infection. Error bars are standard error of the mean (SEM). Data are representative of two independent experiments. Data were analysed using Microsoft Excel v14.0.7140.5002.
Fig 6.4a-6.4c: Bacterial Loads in Urine. Data shows the presence of bacteria (ABSA 834 and ABSA 834maeE mutant) in mouse urine at 6h, 12h and 24h post infection in CFU/ml. Average values are calculated from ten mice (per group). Data are representative of two independent experiments. Error bars are calculated as SEM. Data were analysed using Microsoft Excel v14.0.7140.5002.
Immune response analysis

To analyse the host immune response to different ABSA and UPSA isolates, expression of 23 different cytokines and chemokines was examined in mouse bladders at 24h post infection using Bio-Plex kits (Bio-Rad Laboratories, Australia). There were several different patterns of production observed in the response to infection comparing the different cytokines and chemokines that were analyzed and the different infection conditions. These patterns included (i) factors that were unchanged compared to controls, (ii) upregulated compared to controls, (iii) different between ABSA and UPSA, and (iv) different between WT ABSA 834 and its maeE-deficient mutant.

For example, some of the cytokines such as IL-5 and IL-9 (Fig 6.5a and 6.5b, respectively) showed no statistically significant change in the expression level comparing the infected group with the control (non-infected) mice; for IL-5 for example, this could be seen for ABSA 1014 or UPSA 807 and control; for IL-9, for example comparing UPSA 807 and control. In contrast, other cytokines such as IL-10 (Fig 6.6a) and IL-17A (Fig 6.6b) exhibited significantly higher levels of expression in response to infection with either ABSA or UPSA compared with the control mice that received only PBS. A Table of statistical comparisons is provided below (Table 6.2).
**Fig 6.5a-6.5b: Immune Response Analysis** The graphs illustrate quantification of cytokines IL-5 (5a) and IL-9 (5b) in response to PBS control (●), ABSA 834 (■), ABSA 834maeE (▲), ABSA 1014 (▼) and UPSA 807 (♦) groups. (5a) Pairwise comparisons of interest were ABSA 1014 or UPSA 807 with PBS control. (5b) Pairwise comparisons of interest were PBS control and UPSA 807. Data points represent twenty mice in each infected or control group from two independent experiments with mean calculated. No asterix = P>0.1, *=P<0.1, **=P<0.05, ***=P<0.005. Statistical analyses were performed using GraphPad Prism v5 and SPSS v22.0.

**Fig 6.6a: IL-10**

![IL-10 Graph](image1.png)

**Fig 6.6b: IL-17A**

![IL-17A Graph](image2.png)

**Fig 6.6a-6.6b: Immune Response Analysis** The graphs show statistical significance in production of IL-10 (6a) and IL-17A (6b) in response to PBS control (●), ABSA 834 (■), ABSA 834maeE (▲), ABSA 1014 (▼) and UPSA 807 (♦) groups. Pairwise comparisons of interest were ABSA 1014 or UPSA 807 when compared to control group. Data points represent twenty mice in each infected or control group from two independent experiments with mean calculated. No asterix = P>0.1, *=P<0.1, **=P<0.05, ***=P<0.005. Statistical analyses were performed using GraphPad Prism v5 and SPSS v22.0.
Comparisons of bladder immune responses of mice infected with ABSA 1014 and UPSA 807 showed several notable differences. For example, there was significantly higher production of Eotaxin and MIP-1α in the response to ABSA 1014 compared to UPSA 807 (Fig 6.7a and 6.7b), respectively. Our analysis to compare the effect of maeE deficiency in ABSA 834 towards host immune responses demonstrated that the absence of the maeE gene caused infected bladders to produce significantly lower levels of some cytokines such as IL-1α and MIP-1β when compared to WT ABSA 834 (Fig 6.8a and 6.8b, respectively). A Table of statistical comparisons is provided below (Table 6.2). All other data related to cytokines and chemokines are shown in supplementary figures S1a-S1o, Appendix 3.

![Fig 6.7a: Eotaxin](image1)

**Fig 6.7a: Eotaxin** The graphs show statistical significance in production of Eotaxin (7a) and MIP-1α (7b) in response to PBS control (●), ABSA 834 (■), ABSA 834maeE (▲), ABSA 1014 (▼) and UPSA 807 (♦) groups. Pairwise comparisons of interest were ABSA 1014 infection compared to UPSA 807. Data points represent twenty mice in each infected or control group from two independent experiments with mean calculated. No asterix =P>0.1, *=P<0.1, **=P<0.05, ***=P<0.005. Statistical analyses were performed using GraphPad Prismv5 and SPSSv22.0.

![Fig 6.7b: MIP-1α](image2)

**Fig 6.7b: MIP-1α**
**Fig 6.8a-6.8b: Immune Response Analysis** The graphs show statistical significance of IL-1α (8a) and MIP-1β (8b) in response to PBS control (●), ABSA 834 (■), ABSA 834maeE (▲), ABSA 1014 (▼) and UPSA 807 (♦) groups. Pairwise comparisons of interest were ABSA 834 and ABSA 834maeE mutant infection. Data points represent twenty mice in each infected or control group from two independent experiments with mean calculated. No asterix =P>0.1, *=P<0.1, **=P<0.05, ***=P<0.005. Statistical analyses were performed using GraphPad Prism v5 and SPSS v22.0.
Table 6.2: Statistical comparison table

<table>
<thead>
<tr>
<th>Comparison</th>
<th>IL-5</th>
<th>IL-9</th>
<th>IL-10</th>
<th>IL-17A</th>
<th>Eotaxin</th>
<th>MIP-1α</th>
<th>IL-1α</th>
<th>MIP-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl and ABSA 834</td>
<td>P=0.004</td>
<td>P=0.000</td>
<td>P=0.000</td>
<td>P=0.000</td>
<td>P=0.055</td>
<td>P=0.000</td>
<td>P=0.000</td>
<td>P=0.000</td>
</tr>
<tr>
<td></td>
<td>Δ=1.8</td>
<td>Δ=1.1</td>
<td>Δ=2.8</td>
<td>Δ=38.5</td>
<td>Δ=N/A</td>
<td>Δ=3.4</td>
<td>Δ=69.4</td>
<td>Δ=3.1</td>
</tr>
<tr>
<td>Ctrl and ABSA 834 (\Delta maeE)</td>
<td>P=0.316</td>
<td>P=0.128</td>
<td>P=0.007</td>
<td>P=0.001</td>
<td>P=0.102</td>
<td>P=0.021</td>
<td>P=0.005</td>
<td>P=0.008</td>
</tr>
<tr>
<td></td>
<td>Δ=1.3</td>
<td>Δ=1.0</td>
<td>Δ=1.9</td>
<td>Δ=21.4</td>
<td>Δ=N/A</td>
<td>Δ=2.4</td>
<td>Δ=44.8</td>
<td>Δ=2.0</td>
</tr>
<tr>
<td>Ctrl and ABSA 1014</td>
<td>P=0.133</td>
<td>P=0.007</td>
<td>P=0.000</td>
<td>P=0.000</td>
<td>P=0.026</td>
<td>P=0.000</td>
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</tr>
<tr>
<td></td>
<td>Δ=1.3</td>
<td>Δ=1.0</td>
<td>Δ=2.3</td>
<td>Δ=36.1</td>
<td>Δ=N/A</td>
<td>Δ=3.6</td>
<td>Δ=86.0</td>
<td>Δ=2.8</td>
</tr>
<tr>
<td>Ctrl and UPSA 807</td>
<td>P=0.222</td>
<td>P=0.728</td>
<td>P=0.003</td>
<td>P=0.000</td>
<td>N.D.</td>
<td>P=0.000</td>
<td>P=0.000</td>
<td>P=0.002</td>
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<td></td>
<td>Δ=1.3</td>
<td>Δ=0.9</td>
<td>Δ=2.2</td>
<td>Δ=38.4</td>
<td>Δ=N/A</td>
<td>Δ=2.5</td>
<td>Δ=65.0</td>
<td>Δ=2.5</td>
</tr>
<tr>
<td>ABSA 834 and ABSA 834 (\Delta maeE)</td>
<td>P=0.067</td>
<td>P=0.033</td>
<td>P=0.010</td>
<td>P=0.006</td>
<td>P=0.700</td>
<td>P=0.070</td>
<td>P=0.098</td>
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<td>Δ=1.4</td>
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<td>Δ=1.5</td>
<td>Δ=1.8</td>
<td>Δ=1.3</td>
<td>Δ=1.4</td>
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<tr>
<td>ABSA 1014 and UPSA 807</td>
<td>P=0.665</td>
<td>P=0.191</td>
<td>P=0.675</td>
<td>P=0.735</td>
<td>P=0.026</td>
<td>P=0.059</td>
<td>P=0.256</td>
<td>P=0.464</td>
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<tr>
<td></td>
<td>Δ=1.0</td>
<td>Δ=1.1</td>
<td>Δ=1.0</td>
<td>Δ=0.9</td>
<td>Δ=N/A</td>
<td>Δ=1.4</td>
<td>Δ=1.3</td>
<td>Δ=1.1</td>
</tr>
</tbody>
</table>

Shaded box represents the pair-wise comparisons of cytokines of interest and discussed in text. P values were calculated using SPSS v22, Independent Sample T-Test. N.D.: P value cannot be computed because the standard deviation of both group are zero. Δ: represents fold-change in the denominator condition listed in the Comparison Column compared to the numerator condition.
Discussion

Depending on the symptoms, UTI ranges from acute or symptomatic UTI to ABU. Symptomatic UTI can originate from bladder (cystitis) or kidney (pyelonephritis) and can lead to infections in blood (sepsis) (16, 99, 103, 490, 491). Pathogens that cause symptomatic UTI are referred to as uropathogens, whereas microbes that can establish bacteriuria with significant counts of bacteria in the urine without being associated with any one of the multiple 'classic' UTI symptoms (256), are classified as ABU organisms. Effective host cell surface attachment is considered an important virulence characteristic of most uropathogens but has not been widely associated with strain of bacteria that cause ABU. This host cell surface attachment of uropathogenic bacteria to host cells appears to be critical for the successful establishment of infection in the bladder and underpins colonization for UTI (261, 491). Thus, symptomatic UTI is associated with bacteria that can express multiple adhesins that can promote this virulence characteristic, and UPEC provides a good example of this (492, 493). The presence of high numbers (>10^5 CFU/ml) of bacteria in the urinary tract or bladder in patients without any symptoms in the form of ABU is a common type of infection but it is not well understood. For instance, ABU occurs in an estimated minimum 1% of the population, that carries >10^5 CFU/ml of bacteria in their urine for months or years without any symptoms (325). The mechanisms of how ABU isolates persist in the hydrodynamic environment of the human bladder, and specifically, in urine are largely unknown and have not been investigated for S. agalactiae. Furthermore, how ABSA colonize and persist in the bladder (perhaps only in the urine) without provoking the host immune system that can contribute to symptoms (141) is not clear. The work in this Chapter was aimed at studying the ability of ABSA and UPSA isolates to colonize and persist in the bladder in vivo using transurethral infected C57BL/6 female mice as a model of human UTI. We analysed the impact of the maeE gene on colonization based on data in Chapter 5. Finally, this study also evaluated the immune response to ABSA and UPSA isolates during these infections in mice by measuring the levels of chemokines and cytokines in the bladder.
A major finding of this Chapter was that ABSA 1014 colonized the bladders of mice significantly better than UPSA 807, when assessed using colony counts of viable bacteria at 24h post infection. When we assessed colony counts of viable bacteria in the urine of mice at 24h post infection, ABSA 1014 was also recovered in higher numbers at 6h and 24h compared to UPSA 807. Thus, the data related to urine collected from mice infected with ABSA 1014 and UPSA 807 were consistent with the data related to tissue colonisation. This is an interesting result, which strongly supports the previous findings reported in Chapter 4 and 5 where it was shown that ABSA isolates can out-compete UPSA isolates in human urine. The findings from the current Chapter agree with these observations and show that ABSA 1014 can colonize the urine of mice better than UPSA 807. Collectively, these data indicate that ABSA can grow better in the nutrient limited and acidic environment of urine. The findings from the current Chapter also supports several other studies reported in the literature regarding the ability of bacteria recovered from individuals with asymptomatic bacteriuria to survive better in the host urinary tract compared to uropathogenic isolates. For example, prototypic *E. coli* 83972, a ‘prototype’ ABU strain, grows better in urine that some UPEC (261). The strain has been used for the establishment of long-term urine colonization in spinal cord injury patients for up to 40 months. In a clinical trial, these individuals showed a reduced incidence of UTI with other organisms (175, 494). Some ABU strains have also been reported to be better biofilm formers compared to uropathogenic strains (386).

Biofilm formation is considered as a virulence trait of many Gram negative and Gram positive bacteria including *Staphylococcus epidermidis, S. aureus* (495, 496), *E. faecalis* (497, 498), *Streptococcus salivarius* (499), *S. saprophyticus* (500) and many other oral streptococci, such as *S. mutans* and *S. gordonii* (501). Typically, in UTIs, biofilm formation has been linked to enhanced colonization and bacterial survival because it can help the bacteria resist antibacterial mechanisms (502). There has been little research on biofilm formation by *S. agalactiae*, and none performed on UPSA or ABSA. However, literature has demonstrated
that biofilm formation by *S. agalactiae* is dependent on environmental pH. In the normal condition, the pH of the genitourinary tract is more of an acidic environment (pH 3.8-4.5). *S. agalactiae* strains isolated from the genital tract of asymptomatic women have been reported to form biofilms preferentially in acidic conditions and grow well at pH 5.0-7.0. For such strains, biofilm formation was higher at pH 4.5 than pH 7.0 (501, 503, 504). Furthermore, the ability to form biofilms by *S. agalactiae* was assessed in another study that revealed that 76.5% of genital tract isolates from asymptomatic women were significantly better biofilm producers compared to 36.6% of isolates recovered from symptomatic patients with acute disease (501). Thus it can be argued that the ABSA isolates in this study may be better biofilm producers than UPSA strains, and may be more adapted to acidic environments like urine (with a pH ranges between 4.5 and 6.5).

Generally, if we use *E. coli* as a model example, ABU isolates are considered less virulent and non-adherent to host uroepithelial cells compared to uropathogenic isolates (268, 491, 505, 506). In one study, the adhesin phenotype of some ABU *E. coli* showed that out of nine ABU strains tested, expression of Type 1 and F1C fimbriae seems to be common, but P fimbriae expression was rare, indicating an absence of a major adhesin amongst multiple ABU *E. coli* (268). For the extensively studied *E. coli* ABU strain 83972, which is unable express any functional adhesins, the bacteria can establish successful long-term colonization in the human urinary tract (including urine) and grow in high numbers in urine (261). High numbers of *S. agalactiae* were recovered from several ABU patients (16, 43) who carried these ABU *S. agalactiae* for several months; nothing is known about the adhesin repertoire of these strains. The mechanism(s) of how ABSA isolates colonize the urinary tract for long periods of time despite the hydrodynamic flow of urine is not clear yet. No studies have been conducted to define the adhesin repertoire among these ABU *S. agalactiae* isolates and characterise the expression of these adhesins. A major finding of this thesis is the demonstration that these strains can grow in human urine, which represents a fitness trait independent of adhesins.
This may afford strong colonization potential to these strains in the bladder by virtue of persistence in urine. Interestingly, it is worthwhile to note that this trait is not confined to ABU 83972 but it is widely distributed among other ABU isolates (268). On the other hand, the ability of uropathogens to cause symptomatic UTI has been associated with the expression of several virulence factors including adhesins (141).

Growth of bacteria in urine is linked to the nutritional resources available in urine such as iron (261, 278). Some bacteria possess siderophore biosynthesis for iron acquisition to enable this (278, 361) which contributes to bacterial pathogenicity in general (507, 508). Experiments related to iron utilization by *S. agalactiae* in urine are discussed in Chapter 7. Here, we will discuss the ability of *S. agalactiae* to utilize another constituent in urine, L-malic acid because of the data in this Chapter related to recovery of different *S. agalactiae* strains from mouse urine. No published studies have reported an ability of bacteria to utilize malic acid in human urine. In our study, we discovered that there was an inability of UPSA 807 to utilize L-malic acid, which probably is due to a frame-shift mutation (a single nucleotide, adenine deletion) that we detected in a poly A sequence at the 5’ end of the *maeK* gene of UPSA 807. The genome analysis that identified this mutation and the related phenotype of a lack of malic acid utilization was confirmed by PM arrays as described in Chapter 5. These interesting findings led us to perform *in vivo* assays to further this work, and specifically, undertake bladder colonization experiments with malate pathway-deficient ABSA mutant. In bladder colonization experiments with WT ABSA 834 and a ABSA 834Δ*maeE* mutant we revealed that WT ABSA 834 was significantly (p=0.042) better at colonizing bladder than the ABSA 834Δ*maeE* mutant strain.

Colony counts of urine collected from the mice showed no difference in bacterial recovery between the groups at any of the time points tested (6h, 12h and 24h). However, the colonization ability of WT ABSA 834 over its *maeE* mutant derivative was interesting. There
were significantly higher counts of WT ABSA 834 recovered from bladder after 24h post infection compared to the mutant. This may be because the presence of maeE influenced the ability of WT ABSA 834 to colonise the bladder and enabled it to colonize more efficiently. This can be stated because the data showed that there was significantly fewer numbers of the mutant recovered from the bladder at 24h post infection. Moreover, in the prior Chapters (4 and 5) we demonstrated that a functionally active ME pathway in WT ABSA contributes significantly to a robust urine growth phenotype. L-Malic acid is a organic acid in many fruits and vegetables, is synthesized in kidney cells and is often present in normal human urine in varying amount depending on dietary intake (281, 399). In lactic acid bacteria including L. casei (289) and S. mutans (404, 405) L-malic acid is converted to L-lactate using malo-lactic enzyme. Organisms such as E. faecalis (400, 406), S. bovis (407), and L. casei (289) metabolize L-malic acid to pyruvate using the ME pathway. Our data suggest that the reason behind WT ABSA being recovered in higher number from mice may be that malic acid metabolism contributes to colonisation in some unknown way. It could be that ABSA can utilize available energy sources such as L-malic acid from the nutrient limited environment of urine, in a way similar to that used by the other above mentioned organisms. To our knowledge, this is the first description that a bacteria’s ability to metabolize malic acid has a role in mouse bladder colonization (this study) and human urine utilization (Chapter 5). In a prior study, theoretical and experimental analyses of bacterial growth in bladder suggested that adhesin-mediated surface growth (e.g. of uropathogens) may not be required for bacteria to persist in this host niche. Instead, the microbes ability to grow faster and attain optimal growth by utilizing available energy source in human urine was stated as the key factor for bacterial persistence (267, 384). Our data on urine growth of ABSA are consistent with this notion. Interestingly, the average generation time of WT ABSA 834 was 145min (calculated between 0-36h), compared to ABSA 834ΔmaeE mutant that grew poorly with generation times >600min (two-sample two-tailed t test, P<0.001). Thus, we conclude that efficient utilization of available resources in nutrient limited media like urine is an advantage of WT ABSA isolates over UPSA.
Comparing ABSA 834 with the maeE mutant of ABSA 834 showed that the results generated in human urine ex vivo (i.e. suggesting utilization of the malic acid pathways was important for growth) did not correlate with the mouse bladder colonisation model in which the maeE mutant did not provide a significant difference in the ability to growth in mouse urine in vivo. There could be several reason of this difference. For example, the constitution of mouse urine and human urine would be different and related to the fact that dietary consumption of human and mice is very different (281, 509). Urine constituents largely depend on dietary intake. Mice that are used for experimental purposes are fed on a standard diet of protein chow that would differ to most human diets. Therefore, human urine constituents would vary widely because of a much higher variation in dietary intake. Studies have shown that human urine contains malic acid, sugars and other carbon sources (281, 399). L-Malic acid is a widespread organic acid found at especially high levels in various fruits such as black currants, rhubarb, green apples, grape musts etc (404, 410). The optimal pH of 5.8 to 6.2 (used in this study) for the growth of S. agalactiae in a nutrient limited media like urine is another factor that may impact the differences noted above since bacterial metabolism to achieve normal growth and survival depends on a suitable pH. In one study which involved testing of the pH of mouse urine the researchers showed an average pH of 7.51 (510). This is very different to the average pH range of human urine (5.8-6.2) (352, 358). Reisinger A. J. et al., showed that mice fed a diet supplemented with 1% NH₄Cl exhibited acidified urine (510), which shows that dietary intake plays a major role in the constitution of urine. Considering these differences in pH and constitution of urine, it can be argued that the results of Chapters 5 and 6 could be different due to differences in urine constituency. Liquid chromatography of mice and human urine samples which were used for urine growth experiments in vivo and in vitro could be a useful approach to study these differences between mice and human urine samples. It is also worth noting that experiments conducted ex vivo (Chapter 5) cannot incorporate host immune responses, which would have a large impact on the number of bacteria in the urinary tract in mouse (Chapter 6). It can be argued therefore that differences in the presence of a host immune response could explain differences in the data in Chapters 5 and 6.
For these kind of experiments it would be useful to understand the generation time of the bacteria \textit{in vivo}. To obtain bacterial counts from bladder, mice are sacrificed and the tissues used for colony counts. Another set of ten mice per group in the experiment would allow sacrifice of mice at each time points (6h, 12h and 24h) and perform colony counts. These counts could be used to calculate differences in generation time between WT and \textit{maeE} mutant. However, a limitation of this assay approach is that the amount of killing of the bacteria, which also occurs in the mouse over time, is not taken into account. In addition, the dynamics of voiding of bacteria in the urine is a factor that would confound any attempts at calculation of generation time \textit{in vivo}. So, while experiments could be done over time to calculate the number of bacteria in the tissue and urine the data generated from such assays would need to be interpreted with the view of these confounding, limiting factors that would effect any concept of generation time \textit{in vivo}.

This Chapter also investigated the host immune response to ABSA and UPSA. In response to bacterial bladder colonization and expression of bacterial virulence factors the host immune system produces a range of inflammatory cytokines and chemokines (511, 512). Production of cytokines and chemokines is one of the main protective immune responses of the host to any pathogen. Many bacterial species including uropathogenic bacteria such as UPEC possess the ability to modulate host immune responses and this may allow them to persist in the host niche (513, 514). Recently, \textit{S. agalactiae} had been also reported to have the ability to modulate host immune response (103). The sialic acid residues of capsular polysaccharide of \textit{S. agalactiae}, which are indistinguishable from some host cell surface moieties, are also able to prevent phagocytosis and suppress the neutrophil oxidative burst (102-104, 515). Kline \textit{et al.}, reported that \textit{S. agalactiae} expressing sialic acid with minimal O-acetylation were capable of suppressing the neutrophil oxidative burst and survived better in the urinary tract of C3H/HeN mice than \textit{S. agalactiae} expressing hyper O-acetylation of sialic acid (326). This
study also revealed that capsule of *S. agalactiae* (co-inoculated with UPEC in murine macrophage *in vitro*) contributes to LPS tolerance via suppression of NF-κB driven response.

In our study, we measured a panel of 23 cytokines and chemokines in supernatants from bladder tissue collected from C57BL/6 mice that were challenged with ABSA or UPSA. Several of the cytokines that were analysed, for example IL-5 and IL-9 (Fig 5a and 5b), showed no change in expression level when compared to the non-infected control group. The trend of no change was especially obvious in the case of IL-9 induction. In contrast, other cytokines such as IL-10 and IL-17A (Fig 6a and 6b) showed high levels of expression in most infected groups (ABSA 1014, 834, 834 *maeE* mutant and UPSA 807) when compared to control group. We showed that some of the cytokines induced by *S. agalactiae* are potent regulatory factors that can suppress host immune responses and modulate immune reactivity. Other studies have shown that other uropathogenic bacteria including UPEC can modulate host immune response in bladder and suppress TNF-α mediated IL-8 and IL-6 secretion from urothelial cell cultures *in vitro* (516, 517). In our data, this response was most obvious for IL-10. IL-10 expression in response to ABSA 1014 and ABSA 834 is worthwhile to note because prior studies recognized IL-10 as immunosuppressive and an anti-inflammatory cytokine produced in bladder in response to UTI (315, 518). This suggests that ABSA may induce an immunosuppressive or anti-inflammatory response in humans under some conditions. In the current study, high numbers of ABSA 1014 were recovered from bladders at 24h and it is interesting to compare these bacterial loads with the cytokine response. In terms of the IL-10 response, the current data are consistent with data from Kline *et al.*, who showed an immune suppressive reaction to *S. agalactiae* in the bladder (103). Recent comparison of UPEC infection in bladder (i.e. cystitis) versus kidney (i.e. pyelonephritis) demonstrated that bacterial persistence was observed in bladder at several weeks post infection but bacterial loads in kidneys were cleared after 5 days and IL-10 had a role in this model (519). Chan *et al.*, also demonstrated that localized production of IL-10 results in suppressed humoral and cell
mediated responses which leads to increased bacterial persistence (519). Madureira et al., demonstrated that IL-10-deficient mice were more resistant to S. agalactiae infection and a S. agalactiae strain over expressing GAPDH showed increased virulence in C57BL/6 mice with the presence high concentrations of IL-10 in serum (520). However, it should be noted that this model was not related to UTI and so cannot be directly compared to this work. In another study, S. agalactiae was reported to stimulate IL-10 production in the bladder (103), however even though this study was referring to uropathogenic S. agalactiae, the study used S. agalactiae strain COH1, which is not known to be uropathogenic; this strain was isolated from an infected new-born with sepsis (143, 521). Thus it can be argued that high expression of IL-10 in bladder tissue in our study may contribute in some way to persistence of S. agalactiae in the bladder; this could especially be true for ABSA isolates. In addition, results of gradually reducing numbers of UPSA 807 recovered from urine at each time point could reflect differential induction of specific immune modulatory factors (that we have shown in this series of experiments) that are different between ABSA and UPSA, and are known to influence inflammation and antimicrobial responses. Thus, we speculate that UPSA 807 may modulate distinct immune responses differently compared to ABSA 1014, as shown for other uropathogens such as UPEC.

Another key finding of our immune response analysis of mouse bladder infected with ABSA 1014 and UPSA 807 was that Eotaxin and MIP-1α were produced more in bladders infected with ABSA 1014 compared to UPSA 807 (Fig 7a & 7b). These data are novel because these factors have not readily been described as being produced during infection by S. agalactiae. We also showed that TNF-α was significantly induced in the mice by all of the infection conditions used, which is consistent with prior studies. TNF-α is one of the most known proinflammatory cytokines produced in the bladder during UTI (522). TNF-α production in response to S. agalactiae is considered as an initial response if we look at prior in vitro published data using human cells. Here, researchers have reported that a response of mixed
mononuclear cells to *E. coli* LPS involves the production of TNFα, IL-β, IL-6 and IL-8 simultaneously. In contrast, the response of monocytes to *S. agalactiae* includes the production of TNF-α prior to the appearance of any other cytokines (523). Other cell types such as human lung carcinoma A549 cells also produce TNF-α and other cytokines in response to *S. agalactiae* (524). Several virulence factors produced by *S. agalactiae* such as type specific capsular polysaccharides, lipoteichoic acid and β-hemolysin are able to directly stimulate host cells including monocytes to produce TNF-α and other cytokines (113, 124). Mikamo *et al.*, also showed that there was a significant difference in TNF-α production between different *S. agalactiae* serotypes and the host cell response to purified capsular polysaccharides (524). Using the 5737 bladder cell line *in vitro* and C57Bl/6J female mice *in vivo*, it was shown that infection with WT *S. agalactiae* and a *ΔcylE*-deficient mutant (causing β-hemolysin deficiency) led to significantly higher transcriptional up-regulation of proinflammatory cytokines such as IL-6, TNF-α and IL-1α in mice or cells infected with WT *S. agalactiae* (138). β-hemolysin is considered as one of the main virulence factors of *S. agalactiae*, and is already known to be involved in the invasion of human epithelial cells and in the stimulation of pro-inflammatory cytokines (125, 138). Thus, although β-hemolysin is not known to be involved in adherence to bladder epithelial cells, these data show that this virulence trait is involved in modulating host cell signalling during UTI. This was recently discussed elsewhere (138). It is also interesting to consider the possible mechanisms behind immune suppression during *S. agalactiae* UTI. For example, one study showed that TNF-α controlled type 1 immune activation by suppressing T cell proliferation in mice (525) and we have showed strong production of TNF-α in response to ABSA. Collectively, these data imply that the capsule and the β-hemolysin of *S. agalactiae* impact cytokine responses in host cells and tissues.

High levels of IL-1α in urine may be the result of stimulation of host cells in response to infection and elevated levels of this factor may play a role in protection of the female urinary
tract from infection (526). In addition, detection of IL-1α in urine is considered as a potential diagnostic marker for bacterial cystitis (512). Investigation of the effect of maeE deficiency in ABSA 834 towards bladder immune responses showed significantly lower levels of production of IL-1α and MIP-1β by bladder cells of mice comparing a maeE mutant strain with WT ABSA 834. Our results showing the induction of cytokines including IL-1α and MIP-1β are consistent with two previous studies, which demonstrated a strong induction of IL-1α and MIP-1β upon S. agalactiae bladder colonization (87, 103). Altogether, these results of a lower level of cytokine production in response to the ABSA 834 maeE-deficient mutant (such as for IL-1α and MIP-1β) and lower bladder colonization of this mutant suggest that the malic acid metabolic pathway is essential for optimal survival and persistence of ABSA in the host.
Chapter 7

Asymptomatic bacteriuria due to *Streptococcus agalactiae* is associated with bacterial fitness for growth in human urine independent of iron acquisition

*Supplementary figures in Appendix 4.*

My contribution to this Chapter involved:
Design of experiments and generation of data for all growth experiments, generation of *guaA* mutant in ABSA 834, clinical description of isolates, analysis of the experimental results, and writing of the Chapter.

(Signed) _______________________________ (Date)______________
Deepak S. Ipe

(Countersigned) _______________________________ (Date)______________
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Introduction

Urinary tract infection (UTI) is a serious health problem affecting millions of people each year. *Streptococcus agalactiae*, also known as group B streptococcus, is a Gram-positive commensal organism, colonizing the gastrointestinal and genitourinary tracts of up to 50% of healthy adults (4) *S. agalactiae* is also a leading cause of severe, invasive infection in newborns, pregnant women, and older persons with chronic medical illness (12, 19). *S. agalactiae* is classified into serotypes based on its capsular polysaccharide, a major virulence factor that helps the organism escape from host defence mechanisms (527). So far, ten *S. agalactiae* capsular serotypes have been identified namely La, Ib, and II-IX (as well as non-typeable *S. agalactiae*), which are antigenically and structurally unique (16, 528). Serotypes La, III, and V are responsible for the majority of invasive infections (87).

*S. agalactiae* is a major cause of early- and late-onset disease in neonates (426), which can lead to meningitis (529, 530). *S. agalactiae* also causes various infections in adults including skin and soft tissue infections, UTIs, pneumonia and bacteremia. The overall mortality rate of *S. agalactiae* infection in adults is estimated at 15% or more in the United States (12, 26, 45, 531). In terms of UTI, the spectrum of *S. agalactiae* disease in adults comprises cystitis, pyelonephritis, urethritis, and urosepsis (12, 16, 36). The presence of *S. agalactiae* in the urine in the absence of clinical symptoms, termed *S. agalactiae* asymptomatic bacteriuria (ABU), is the most common form of UTI. In most cases, *S. agalactiae* colonization of the urinary tract is thought to occur by an ascending route from the genital tract, where *S. agalactiae* can persist asymptotically (16). In one study, *S. agalactiae* was cultured from 39% of all cases of symptomatic UTI among nursing home residents >70 years of age (22). Thus, *S. agalactiae* is relatively unique in terms of its ability to cause a spectrum of disease in both newborns and adults as well as asymptomatic infection in the urinary tract.
Bacterial growth in human urine is an important lifestyle trait for UTI and has been described for several uropathogens including *E. coli* and Gram-positive bacteria including staphylococci (261, 263, 267, 278, 381). The ability of these bacteria to survive and efficiently utilize the resources available in urine is considered as an important adaptation strategy for persistence in the urinary tract (263, 267). Growth of bacteria in urine is related to the nutritional resources available in urine such as sugars, proteins, amino acids, and some co-factors including iron (278, 532), as previously discussed in Chapter 3. Due to the paucity of iron available in normal human urine, many human pathogens exhibit siderophore (low-molecular-weight Fe$^{3+}$ chelating compounds) biosynthesis for iron acquisition (361). Siderophore driven acquisition of iron from the host contributes to bacterial pathogenicity in general (507, 508). *E. coli* possess four known siderophore systems. Aerobactin is a hydroxamate siderophore which is produced in high levels and has a higher Fe$^{3+}$ binding stability at low pH compared to high pH (533, 534). Enterobactin is a highly prevalent siderophore in *E. coli* and is produced by both non-pathogenic strains (e.g. K-12) and pathogenic strains (535-537). Salmochelin is another derivative of enterobactin (536) and it is considered as a better siderophore for pathogens compared to enterobactin (538) because siderocalin (neutrophil gelatinase-associated lipocalin) cannot recognize this siderophore and thus can evade host immune response (539). Yersiniabactin is the other virulence-associated phenolate/thiazolidine siderophore, and UPEC strains that are known to express this siderophore to-date also co-express enterobactin. Yersiniabactin is also common in *Enterobacteriaceae* (540) and is a virulence determinant for *Yersinia pestis*, *Klebsiella pneumoniae* and *E. coli* (278, 541-543). Yersiniabactin and Salmochelin are produced maximally under neutral to alkaline conditions of pH ranging from 7.0 to 7.6 (533). Progression of bladder infection to kidney or bloodstream infection by some *E. coli* strains has been associated with carriage of the *fyuA* gene (encoding the yersiniabactin receptor) (301, 544). The study showed that a *fyuA* mutant of ABU *E. coli* strain VR50 exhibited a 92% reduction in biofilm formation in urine flow-cell chambers compared with the wild-type (544). Several studies have shown that ABU isolates express at least one of these four siderophore systems *in vitro* and in UTI patients (261, 361, 532). One of the well-studied
E. coli ABU strain 83972, originally isolated from a young Swedish girl who carried it for 3 years without any symptoms (506) is an isolate capable of long-term bladder colonization without provoking the host immune system (174, 175, 260, 321). Three years represents a substantial number of generation time, which could equate to more than 30,000 generations (261). During this time, the strain might have adapted to the urinary tract niche and lost the ability to express various functional virulence factors. One could speculate that this might involve the important virulence factors such as P fimbriae (212, 262), F1C fimbriae (545) or pore-forming hemolysin (261) although there is no direct evidence of this phenomenon. On the other hand, a key factor that may help with the successful long-term colonization of this strain is the ability to grow rapidly in human urine and the expression of genes involved in metabolism of nutrients available in urine (261). More importantly, this E. coli ABU 83972 strain known to express all the four siderophore systems for the optimal growth in iron limiting human urine (261, 278, 361).

Aside from a hypothesized potential role for iron acquisition and siderophore production in S. agalactiae for growth in human urine, we also hypothesize that other genes may play a role. There are several other genes that are of interest to investigate in this context. One such gene is guaA; this gene encodes guanosine monophosphate (GMP) synthase and part of the polycistronic guaBA operon. guaA is separated from the upstream guaB gene by a 68 base pair (bp) intercistronic region (546). Studies have shown that the presence of guaA is important for the growth of E. coli (CP9 strain) in human urine in vitro; in the absence of guaA E. coli also demonstrated significantly less urovirulence compared to WT E. coli in an in vivo mouse model (270). These findings are consistent with a subsequent study on UPEC which showed bacterial guanine synthesis is important for optimal growth in human urine (384). In addition, it is important to note that a guaA transposon insertional mutant in Lactococcus lactis showed many fold increased acid tolerance compared to the WT in a study conducted to identify the genes involved in acid tolerance response, which is relevant because urine is
typically mildly acidic (547, 548). Experimental analysis also revealed that alteration of the guanine nucleotides biosynthesis flux before a lethal acid challenge leads to the improved bacterial survival implying potential importance of guanine in bacterial survival or growth in urine (548). Studies have also revealed the presence of guanine in human urine (549-551). Finally, using signature-tagged mutagenesis and selection of auxotrophic mutants (mutant bacterial strains which have specific nutritional requirements compared to the parental strains), guaA was identified as responsible for the growth and survival of *S. agalactiae* A909 in the host (552). Thus, collectively the literature to date provides sufficient interest to investigate the role of guaA in *S. agalactiae* for growth in human urine.

The potential fitness of *S. agalactiae* for growth in human urine has not previously been investigated. Results from Chapter 4 and Chapter 5 showed growth of some *S. agalactiae* in urine. In addition, from the literature we know that the well-studied *E. coli* ABU 83972 strain produces all four siderophores to grow in iron-limiting medium. Together, these observations prompted the work in this Chapter. In this study, we tested several selected *S. agalactiae* isolates from adult patients with UTI (16) for their ability to utilize human urine as a growth medium. This chapter investigated (i) the diversity in growth phenotype of *S. agalactiae* strains in human urine using turbidity (via OD600nm readings) and CFU colony counts of cultures, (ii) whether there is any correlation between turbidity and CFU colony counts in cultures of *S. agalactiae* in human urine, (iii) the effect of iron limitation on the growth of ABSA 834 in human urine through the use of the iron chelator 2,2'-dipyridyl to infer whether *S. agalactiae* is dependent on iron for growth in human urine, (iv) the presence of thirteen virulence genes including *fhuD* and *guaA* in ABSA 834, and (V) the role of *guaA* in supporting the growth of ABSA 834 in mildly acidic human urine.
Methods

History of Isolates and Ethics

This study was conducted using *S. agalactiae* isolates and clinical and laboratory data from a single-centered study of *S. agalactiae* UTI in patients at University of Alabama at Birmingham (UAB) Hospital conducted between August-2007 and August-2008 (16). The isolates were grouped according to the clinical condition of patients as either high- (>10⁴ CFU/ml) or low-grade ABU (<10⁴ CFU/ml), or acute UTI, and were used in experiments designed to analyze bacterial fitness in different conditions such as iron-limiting media. Additional isolates and patients who attended the UAB Hospital or surrounding clinics in the subsequent 36-month period were also included in this study. Consequently, the isolates used in this study were representative of patients who presented with positive urine cultures for *S. agalactiae* during the 36-month period between August-2007 and July-2010. The study was performed with approval from and in accordance with the ethical standards of the UAB committee on human experimentation and the Helsinki Declaration (approval X070722011). Ethical approval was also sought from and granted by the Griffith University human research ethics committee (approval MSC/02/11/HREC). The need for specific informed consent was waived by the institutional review board of UAB, and the human research ethics committee of Griffith University. The study subjects were adult patients (>18 yrs) encountered at UAB Hospital and surrounding clinics who underwent clinical and microbiological assessment for UTI because of symptoms indicating infection or as part of routine patient screening. Urine samples were obtained as clean-catch voided or catheterized samples from all patients who underwent assessment for UTI during the study period. This included inpatients, patients that were evaluated in the emergency department, and patients from various University Hospital outpatient clinics. In cases where *S. agalactiae* was cultured from urine (any count, single organism) the medical records for each patient were reviewed for presenting symptoms at the time of sample collection and demographic data were recorded. Potential prognostic indicators were collected by medical record review to encompass, on average, the three-week
period either side of the date when the sample was collected. A provisional diagnosis of acute
*S. agalactiae* UTI was defined by the presence of single-organism *S. agalactiae* bacteruria
(any count; limits of detection, $10^3$ and $\geq 10^5$ CFU/ml) with $\geq 1$ symptom that included dysuria,
increased urinary frequency and/or urgency, fever $>38^\circ$C, flank pain and/or lumbar
tenderness. All *S. agalactiae* isolates that were cultured from urine were identified by colony
morphology on TSA 5% sheep blood agar plates (BD), were tested for catalase, and were
grouped using the Remel PathoDx latex agglutination kit.

In cases where urinalysis (UA) was undertaken as part of the diagnosis, acute UTI was
confirmed on the basis of positive urinary leukocyte esterase and significant pyuria ($\geq 10^7$ white
blood cells/L; non-spun). Hence, the case definition for acute *S. agalactiae* UTI was
symptomatic patients with a count of any level since acute infection has been described in
patients with counts as low as $10^2$ CFU/ml in a prior study (16). Exclusion criteria were the
isolation of multiple organisms from urine, or incomplete medical records or laboratory data.
*S. agalactiae* ABU was defined as single-organism bacteruria of any count, in the absence of UTI
symptoms. This definition was chosen as reasonably inclusive given that *S. agalactiae* urine
counts have been shown to vary considerably in individual patients over several hours and
may persist for extended periods, as reported previously (16). Additional inclusion criteria for
*S. agalactiae* ABU were negative UA laboratory values for leukocyte esterase and pyuria,
where these data were available. All study subjects who satisfied the inclusion criteria were
allocated to one of three infection category groups based on the clinical diagnosis and
laboratory values: high- ($>10^4$ CFU/ml) or low-grade ABU ($<10^4$ CFU/ml), or acute UTI.

**Bacterial Strains**

In this Chapter a total of five *S. agalactiae* isolates were used for growth assays. The bacterial
strains used in this Chapter were ABSA 834, ABSA 939, and ABSA 951, and UPSA 714,
In some growth experiments, the ABU *E. coli* 83972 strain was used to study iron-chelation conditions and the effect on bacterial growth. In addition, two *S. agalactiae* strains, COH1 and A909 were used as reference strains for positive control targets in the virulence gene PCR assays for optimization.

**Collection of Urine**

Human urine for each assays were collected freshly from at least six healthy men and women volunteers who had no recent history of UTI or antibiotic usage (preceding month). In order to ease the filtration process with 0.45µM filter and to remove larger particles, the pooled urine been filtered through a bigger pore size filter system prior to the use of 0.45µM Millipore filter. For this, Buchner vacuum flask vacuum filtration method with the aid of Millipore glass filter assembly (Millipore-XX1004700) and a bigger pore sized, 47mm filter paper (Filtech-0282-047) been used. Fresh urine was used within 48h of collection and sterilization or discarded. Ethical approval for collection of urine from adult volunteers was granted by the Griffith University human research ethics committee (approval MSC/11/10/HREC).

**Urine Growth Assays**

For all experiments, individual isolates were streaked on Tryptone Soya Agar (TSA; Oxoid, Hampshire, United Kingdom) supplemented with 5% horse blood (Oxoid, Thermo Scientific, Adelaide, Australia) and incubated at 37°C overnight. Broth cultures of *S. agalactiae* isolates were then prepared in Todd-Hewitt Broth (THB) with shaking (200 rpm) at 37°C overnight. For urine growth assays, fresh overnight broth cultures were back diluted (1:1000) in PBS pH 7.2 and 2 µl were used to inoculate filter sterilized, pooled human urine (200 µl). The growth of all isolates was also examined in rich THB media to determine whether isolates were defective for general growth. For measurement of growth in THB, bacterial cells were seeded at the
same cell number as in urine growth assays. Duplicate cultures were grown in flat-bottom 96-well plates (Cat. No. 82.1581.001-SARSTEDT, Ingle Farm, Australia) at 37°C, shaking (200rpm). Optical density at 600nm (OD 600nm) was recorded at 0-72h (2h intervals until 16h, then at 24h and 12h intervals thereafter) using a POLARstar Omega BMG Labtech plate reader. Duplicate wells containing sterile urine or THB were used as negative controls. The number of viable S. agalactiae present in cultures over the time course was determined at selected intervals (6h until 12h, then at 24h and 12h thereafter) by colony counts on TSA with 5% defibrinated horse blood. All growth experiments were repeated three times and data are shown as CFU/ml from one experiment, representative of several.

Uropathogenic bacteria such as E. coli have been reported to utilize iron for growth in human urine (278) and we next sought to determine whether S. agalactiae growth in urine might be dependent on iron acquisition. For this, growth assays in human urine were conducted essentially as described above using iron-limiting conditions. Briefly, sterilized urine or THB was supplemented with 200µM 2,2'-dipyridyl (Cat. No: D216305, Sigma-Aldrich, New South Wales, Australia), which is a common iron chelator used to establish iron-limiting conditions (278, 553). S. agalactiae iron dependency was examined by comparing the growth of isolates in iron-limiting media with normal media. E. coli strain 83972, which is known to be dependent on iron for growth in human urine (278), was used as a control.

PCR Screen for S. agalactiae Growth Genes

S. agalactiae utilizes several transport systems and metabolic pathways for growth (380, 554) and we decided to screen a selection of S. agalactiae isolates that displayed robust growth in urine for thirteen virulence genes including those encoding siderophores known to be involved in pathogenicity. These included those listed in Table 7.1 below. We used PCR and a series of gene-specific primers to detect the presence of these genes. Primer sequences used to
screen all 13 genes are listed in Table 7.2 below and PCR conditions were optimized for both MgCl₂ and annealing temperature, which are also listed below in Table 7.2 for each primer pairs using reference strains COH1 and A909. PCR conditions were: Initial denaturation at 95°C for 2min, then 35 cycles of denaturation at 94°C for 50sec, annealing at 55°C for 50sec, extension at 72°C for 1min and final extension at 72°C for 4min. PCR products (typically 20 microliters) were analyzed by gel electrophoresis using 1.2% agarose gel at 110 volts.

Table 7.1: Selected growth genes screened in this Chapter and their functions

<table>
<thead>
<tr>
<th>Selected Genes</th>
<th>Hypothesized Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>fhuD</em></td>
<td>Ferrichrome ABC transporter substrate-binding protein, encodes proteins that participate in siderophore (hydroxamate)-dependent iron(III) transport in other bacterial species</td>
<td>(143, 555)</td>
</tr>
<tr>
<td><em>cydA</em></td>
<td>Subunit of cytochrome bd quinol oxidase; involved in respiration</td>
<td>(556)</td>
</tr>
<tr>
<td><em>dppA</em></td>
<td>ABC transporters for dipeptides. Dipeptide permease subunit A; substrate-binding membrane associated lipoprotein</td>
<td>(552, 557)</td>
</tr>
<tr>
<td><em>dppB</em></td>
<td>ABC transporters for dipeptides</td>
<td>(557)</td>
</tr>
<tr>
<td><em>oppA1</em></td>
<td>OppA – OppF: ABC transporters for oligopeptides</td>
<td>(380, 557)</td>
</tr>
<tr>
<td><em>oppA2</em></td>
<td>Same As Above</td>
<td>(557)</td>
</tr>
<tr>
<td><em>oppB</em></td>
<td>Same As Above</td>
<td>(380, 557)</td>
</tr>
<tr>
<td><em>oppC</em></td>
<td>Same As Above</td>
<td>(380, 557)</td>
</tr>
<tr>
<td><em>oppD</em></td>
<td>Same As Above</td>
<td>(380, 557)</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>oppF</td>
<td>Same As Above</td>
<td>(380, 557)</td>
</tr>
<tr>
<td>dspA</td>
<td>Encodes dipeptide symporter</td>
<td>(557)</td>
</tr>
<tr>
<td>braB</td>
<td>Involved in branched-chain amino acid transport</td>
<td>(380)</td>
</tr>
<tr>
<td>guaA</td>
<td>GMP synthase, Involved in guanine metabolism</td>
<td>(270, 548, 552, 558)</td>
</tr>
</tbody>
</table>
Table 7.2: Virulence genes screened in this Chapter; details of optimized primer pairs and PCR conditions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pairs (5’ - 3’)</th>
<th>MgCl₂</th>
<th>Annealing Temp.</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
</table>
| *fhuD* | F - TGTCCTCACACTACTGACCTTC  
          R - TTAGTTTCTCCACGCCTGC | 4mM   | 55°C            | 605           |
| *cydA* | F - TGCACCATGATGTCATGTCATC  
          R - GGGCAATGAAGCAGAACAACAAAC | 4mM   | 55°C            | 557           |
| *dppA* | F - GCATTAAGACCTCCTCCTACCT  
          R - GGAGAGAGCAGAAATACCTTAG | 4mM   | 55°C            | 738           |
| *dppB* | F - GAGTTACTCATGACCTTAGGCT  
          R - TCCAACAGGGCTTTCCATAC | 4mM   | 55°C            | 512           |
| *oppA1* | F - GGGCGTCATGTCATAAGGA  
          R - CGGTAGTAGTATTTTGTGTGTACAT | 4mM   | 55°C            | 425           |
| *oppA2* | F - GGCGTGCTATCAAAAGGA  
          R - CTGACCAACCTCAGCAAGAC | 4mM   | 55°C            | 1066          |
| *oppB* | F - CCCTATCGTTTTTCTGATGAGATCC  
          R - CGCCAAAGGGCAGTAGTAA | 4mM   | 55°C            | 650           |
<p>| <em>oppC</em> | F - AGGTGCCAGGTCTTCTAGCA | 4mM   | 55°C            | 628           |</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Concentration</th>
<th>Temperature</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>oppD</td>
<td>F - TGGTGAATTCCACACATATGCTGG</td>
<td>R - CCCCAAGTATATGGATGGTGGA</td>
<td>4mM</td>
<td>55°C</td>
<td>723</td>
</tr>
<tr>
<td>oppF</td>
<td>F - GGGAGGAAAAGCCTAATGAC</td>
<td>R - CGTATAGGGGTGGATTGG</td>
<td>4mM</td>
<td>55°C</td>
<td>764</td>
</tr>
<tr>
<td>dspA</td>
<td>F - CTCAATCACCNGCTAAGCAT</td>
<td>R - CGGAGTACTACTAGCAGAGGAATT</td>
<td>4mM</td>
<td>55°C</td>
<td>278</td>
</tr>
<tr>
<td>braB</td>
<td>F - GGTATCTACTCTGGTTCTAATTGGC</td>
<td>R - GCCGGCAATAGAAAGTTGTTAGACA</td>
<td>4mM</td>
<td>55°C</td>
<td>1089</td>
</tr>
<tr>
<td>guaA</td>
<td>F - ATTCCCACTCAACAGTTGCA</td>
<td>R - ATCGGAAATGAGTTCGTGTA</td>
<td>4mM</td>
<td>55°C</td>
<td>637</td>
</tr>
</tbody>
</table>

**Investigation of the effect of guaA-deficiency in ABSA 834 on growth in human urine**

To analyse the role of guaA in ABSA 834 growth in human urine, a guaA-deficient ABSA 834 mutant was generated essentially in the same way as described in Chapter 5 for maeE. Primers used to generate a guaA-deficient mutant in ABSA 834 were: guaA-U-ampi-F(b2), 5’-ATGCGAGGATCCCAGCTAATGAC-3’ (BamH1), and guaA-U-ampi-R(a), 5’-CCAATTTCCGGGATTTGGAACCTAAAGCAACTGTTGAGTGGGAAATAAAAACCTTAGCTACTTTT-3’; and guaA-D-ampi-F(b), 5’-CTAAATGTCACTACCTGCC-3’, and guaA-D-ampi-R(b3), 5’-GCATTTCGCGCACCAGACCGAT -3’ (NotI), respectively that were designed based on the *S. agalactiae* 2603V/R genome. Underlined bases were
incorporated into primers to produce restriction-endonuclease sites for ligation. The chloramphenicol acetyltransferase (cat) gene was amplified from pLZ12 (395) for cloning and selection using primers Cm-ampli-F, 5'-TTAGTTCAACAAACGAAAATTGG-3', and Cm-ampli-R, 5'-GGGGCAGGTTAGTGACATTAG-3'. The upstream reverse guaA-U-ampli-R(a) and downstream forward guaA-D-ampli-F(b) primers incorporated 5' overhangs complementary to cat (in bold) to facilitate a three-way sewing PCR using re-amplification primers guaA-U-ampli-F(b2) and guaA-D-ampli-R(b3) primers. Amplification reactions were performed in 50μl volumes containing approximately 50ng of DNA, 0.2mM dNTPs, 1.5-4.0mM MgCl₂, 200nmol of each primer, and 1 unit of High Fidelity Phusion DNA polymerase (Thermo Scientific) in the manufacturer's supplied HF Phusion buffer. An initial denaturation for 2min at 98°C was used, followed by 35 cycles each consisting of 10s at 95°C (denaturation), 50s at 65°C (annealing), and 30s/kb at 72°C (extension), with a final extension for 4min at 72°C. The rest of the procedures used to generate, select and confirm the guaA (double cross-over) mutant were same as described in Chapter 5 (generation of ABSA 834ΔmaeE). The PCR approach to confirm the mutant used the following primers designed outside of the target region of the guaA interruption site: GuaA-CM-guaA-GBS-Screen-F, 5'-CGTAAACACGCAAAGGTGGGC-3' (targeting the flanking region of the orfA gene) and GuaA-CM-guaA-GBS-Screen-R, 5'-AGTTTACTGGATGCTTTACGCC-3' (targeting the flanking region of guaB gene). Double cross-over mutants were confirmed with these screening primers which give an amplicon size of 2102bp. PCR conditions were, initial denaturation for 30sec at 95°C was followed by 35 cycles each consisting of 40sec at 94°C (denaturation), 50sec at 60°C (annealing), and 3min at 68°C (extension), with a final extension for 3min at 68°C. Growth experiments were then conducted to compare the growth of WT ABSA 834 and the guaA-deficient ABSA 834 mutant (GU2086) in human urine. Both WT ABSA 834 and ABSA 834ΔguaA were routinely grown in THB simultaneously in these experiments to determine the respective growth patterns in a standard nutrient rich media. Growth assays were performed as described in Chapter 4 and 5.
**Results**

**Growth in Human Urine**

In this Chapter, we studied five *S. agalactiae* isolates for growth experiments, and subsequently we focused on the ABSA 834 ‘fast-grower’ isolate that exhibited robust growth in human urine. Growth was measured using turbidity (Fig 7.1a) and CFU determination (Fig 7.1b). When generation times of ‘fast-grower’ *S. agalactiae* isolates were calculated using CFU counts, we found only minor variations between the times comparing urine and THB (avg. time in urine: 163 min versus 145 min in THB, 0-36h).

![Fig 7.1a: S. agalactiae Growth in Urine; Turbidity](image)

![Fig 7.1b: S. agalactiae Growth in Urine; Colony Count](image)
**Fig 7.1a-7.1b: Growth in Human Urine** Graphs show the growth of *S. agalactiae* isolates ABSA 834 (▲), ABSA 951 (■), ABSA 939 (●) and UPSA 807 (▲) in human urine. Fig 7.1a demonstrates average of duplicate turbidity measurement at OD 600nm of isolates obtained from two independent experiments. Error bars are denoting the standard deviation of two data points. Fig 7.1b demonstrates average of colony counts (CFU/ml) of isolates obtained from two independent experiments. Error bars denote the standard deviation of the two data points. Data analyses were performed using Microsoft Excel v14.0.7140.5002.

We also aimed to investigate the relationship between *S. agalactiae* culture turbidity and viable cell count overtime (0h–72h). We achieved this by comparing absorbance readings at OD 600nm for turbidity measures with CFU counts in each experiment (Fig 7.2). These assays studying the relationship between turbidity and viable cell count also made use of the data generated in Chapter 4 based on 358 *S. agalactiae* isolates to infer any potential relationship between these measures by calculating an R$^2$ value. Curiously, the results demonstrated that overall, there was a poor correlation between turbidity and viable cell count (average R$^2$ 0.32 n=358). However, there was considerable variation between individual isolates. For example, isolate 951 showed fast growth according to OD600nm and colony counts with good correlation between both measures (R$^2$ 0.86; Fig 7.2). In contrast, isolate 42 demonstrated poor correlation between OD 600nm and viable colony count data (R$^2$ 0.31; Fig 7.2).
Figure 7.2: Correlation of Colony Count and OD600nm measurements

Figure 7.2 demonstrates comparison between turbidity measurements and colony counts of ABSA 951 and UPSA 42. Each data point represents average of duplicate turbidity measurement at OD 600nm and colony count from at least two independent experiments. Error bars denote the standard deviation of the two data points. Data analyses were performed using Microsoft Excel v14.0.7140.5002.
Effect of Iron limitation on growth of *S. agalactiae* in urine

The growth of ABSA isolates was determined in human urine with and without 200µM 2,2’-dipyridyl (an iron chelator) to reveal whether iron availability in human urine plays a role in the optimal growth of selected ABSA strains. The results demonstrated that the growth of ABSA isolates in urine with the presence or absence of the iron chelator 2,2’-dipyridyl was similar (Fig 7.3, ABSA 834: representative of other ABSA isolates tested). These results were compared to a reference control ABU *E. coli* strain 83972, which was grown in the same batch of urine simultaneously. These experiments showed that the presence of the 2,2’-dipyridyl significantly inhibited the growth of ABU *E. coli* strain 83972 in human, indicating that iron limitation impaired the growth of *E. coli*. This finding was in contrast to the absence of any effect of 2,2’-dipyridyl on ABSA cultures, which had very similar growth curves compared to control ABSA cultures that did not contain 2,2’-dipyridyl (Fig 7.3).

**Fig 7.3: Effect of iron on Growth of *S. agalactiae* in Human Urine**
**Fig 7.3: Effect of Iron on Growth of S. agalactiae in Human Urine**

Graphs show the effect of iron depletion on growth of ABSA in human urine supplied with and without 200µM 2,2'-dipyridyl when compared to ABU *E. coli* strain 83972. Each data point at each time point represents average value of at least two independent experiments. Error bars denote the standard error of the mean (SEM). Data analyses were performed using Microsoft Excel v14.0.7140.5002.

**PCR detection of virulence related genes including siderophores**

In this Chapter, we used PCR to determine the genetic makeup of ABSA 834 in terms of the genes encoding virulence factors that might impact the growth in urine including siderophores. Initially, PCR conditions for thirteen genes were optimized for primers, annealing temperature, and MgCl$_2$ concentrations to establish robust techniques for detecting the genes in reference strains, COH1 and A909. These results showed that all the selected thirteen genes were present in reference strain COH1 (Fig 7.4a) and A909 (Appendix 4, Supplementary Fig S1a-S1c). We then analysed ABSA 834 for the presence of these genes. The results showed that all the thirteen genes (Table 7.1) were also present in ABSA 834 (Fig 7.4b and Appendix 4, Supplementary Fig S1d).
Fig 7.4a: Screening of 13 genes in COH1

Fig 7.4a and Fig 7.4b shows presence or absence of thirteen genes in reference strain COH1 (genotype available in NCBI database) and ABSA 834 respectively.

Fig 7.4a-7.4b: PCR screening for the presence of selected genes in S. agalactiae.
The results of comparative growth assays with WT ABSA 834 and ABSA 834ΔguaA in human urine showed that with the deletion of guaA, the normally fast grower ABSA 834 could not grow or survive in human urine compared to WT ABSA 834 (Fig 7.5). Growth assays of WT ABSA 834 and ABSA 834ΔguaA in nutrient rich THB media demonstrated that there was no general growth defect in the ABSA 834ΔguaA mutant (Fig 7.5). In THB, the guaA mutant showed a very similar growth pattern compared to WT ABSA 834.

**Fig 7.5: The effect of guaA-deficiency in ABSA 834 on growth in human urine and THB**

The line graph shows the effect of guaA-deficiency on growth of ABSA 834 in human urine and THB. Each data point at each time point represents average value of at least two independent experiments. Error bars denote the standard error of the mean (SEM). Data analyses were performed using Microsoft Excel v14.0.7140.5002.
Discussion

Previous Chapters showed that human urine can support the growth of some S. agalactiae. However, it is not yet known what are the essential factors that are contained in human urine to support the growth of S. agalactiae and whether iron (or other factors) may play a role in the optimal growth of S. agalactiae in human urine. The primary aims of this study were to investigate (i) growth patterns of S. agalactiae in human urine using turbidity (via OD600nm readings) and CFU colony counts of cultures, (ii) whether there is any correlation between turbidity and CFU colony counts in cultures of S. agalactiae in human urine, (iii) the effect of iron limitation on the growth of ABSA 834 in human urine through the use of the iron chelator 2,2’-dipyridyl to infer whether S. agalactiae is dependent on iron for growth in human urine, (iv) the presence of virulence genes including those encoding siderophores (fhuD) and GMP synthase (guaA) in ABSA 834, and (V) the role of guaA in supporting the growth of ABSA 834 in mildly acidic human urine.

Overall, the urine growth data showed that each S. agalactiae isolate is phenotypically unique in terms of urine utilization, and both measures of turbidity and CFU determination as indicators of growth are important to accurately gauge the fitness of individual S. agalactiae isolates for growth in human urine. We showed that an OD600nm measurement would be more reliable and a robust technique for screening a large number of S. agalactiae isolates for determining their growth in human urine. Growth curve measurements by OD600nm readings are also dependable and useful at the very start of the experiments to record the growth readings even in short intervals such as every two hours of incubation. In this way, we can understand and estimate an overall trend in growth of bacterial species in different culture media such as urine and THB. This technique is useful where bacterial species generation time is unknown in
different media. For example, in this chapter, the fitness and generation time of each ABSA and UPSA isolates in human urine were previously unknown. Thus, initial screening using OD600nm measurement would be a robust and easy technique. However, because of the potential variables such as chain length, structure of the bacterial cell and cell size, clumping, and death of bacterial cells over time (as mentioned in Chapter 4) OD600nm readings may incorporate some inaccuracy in viable bacterial cell counts. The formation of aggregated bacterial buttons at the bottom of the culture plates or tube were also sometimes challenging in estimating the viable count of bacteria through OD600nm readings. In such cases, it was felt that more accurate growth measurements could be achieved using colony counts which would enable the precise recording of colony forming units of viable bacteria per millilitre (CFU/ml). Even though the colony count technique is labour intensive, this technique allows careful pipette-driven mixing of the precipitated aggregated bacterial buttons at the bottom of some culture plates. These manipulations used in the colony count approach are used for OD600nm measurements (e.g. pipetting, dilution, culture plates used). Thus, CFU determination offers a potentially more accurate and reliable growth measure. In our study, we choose to undertake assays to measure all bacterial strains with both techniques in order to compare the approaches, and more fully understand how the data we obtain in both techniques truly reflects the numbers of bacteria in the urine cultures.

Application of both technical approaches to growth measurement revealed the interesting finding that there was not always complete correlation between the readouts from both techniques. In this aspect, the correlation between the techniques seemed to depend somewhat on individual strain. In our growth assays, for example, comparing colony count and OD600nm readings for ABSA 951 showed almost similar results in growth patterns between the two techniques with an estimated correlation $R^2$ value of
Interestingly, UPSA 42 exhibited slow growth according to OD600nm readings but was a fast grower according to colony counts. The correlation between OD600nm reading and colony counts of UPSA 42 was poor with a $R^2$ value 0.31. Altogether our observations suggest that growth data of isolates in urine require both growth measurements to provide the best and most reliable information on growth of individual isolates for further studies. For example, UPSA 42 might be excluded from future studies to enable the use of turbidity measures in future experiments (less labour intensive). For example, in experiments where screening is necessary such as in the testing of transposon mutants a *S. agalactiae* isolate which exhibits a good correlation value ($R^2 \sim 0.9$) with both type of growth measurements would be useful for study.

Iron is required for virulence of different species of bacteria and has been known as an essential factor for bacterial growth for many years (508, 533, 559, 560). The two ionic forms of iron are FeII and FeIII and iron either alone or combined as iron-sulfur or iron-heme. These forms of iron serve as a catalytic center of enzymes and enzymes play major role in cellular processes such as electron transport, amino acid and nucleic acid synthesis and DNA synthesis (561). However, free soluble iron available in the mammalian host is very limited and the available iron is normally complexed with high affinity iron-binding glycoproteins called sequestering proteins such as transferrin and lactoferrin (293). A study on iron lost from the human body through urine was 0.1mg/day or 0.0018mM/day (562) which is extremely low. To compete and scavenge iron in such iron-limited environments bacteria must possess efficient mechanisms to acquire iron. Production of siderophores is one of the mechanisms that most bacteria utilize for this. Siderophores are low molecular weight chelating compounds which have the ability to release iron from transferrin and lactoferrin (555). Gram negative bacteria such as *E. coli* strains 83972 (261, 278) and Nissle 1917 (533) are known to produce all the four types of siderophores for iron uptake. Different species of streptococci have
also been studied with respect to iron acquisition. For example, group A streptococcus (GAS) have been identified with proteins responsible for haemoprotein binding and transport (560). Evans et al., 1986 studied on Streptococcus mutans ferrous iron transport system and showed it encodes a Mn/Fe uptake system (559). Furthermore, Streptococcus pneumonia has different iron transport system which are essential for bacterial virulence (508). Other constituents of urine are also known to influence bacterial siderophore production indicating the regulation of expression may occur in difference environments (563). Interestingly, a role for iron acquisition systems in S. agalactiae growth in human urine has not been investigated to date. In our study, the growth patterns of ABSA 834 in human urine with and without the presence of the iron chelator 2,2’-dipyridyl were similar over time (0h-48h). In contrast, a control bacterial strain known to use iron for growth in urine, ABU E. coli 83972, showed significantly reduced growth in the presence of the iron chelator in fresh human urine when compared to conditions without the iron chelator. This result indicates that availability of iron in human urine does not impact the growth of S. agalactiae (at least ABSA 834) in urine but it does influence the growth of ABU E. coli 83972 as was expected. In other words, this data indicates that S. agalactiae is not dependent on siderophore biosynthesis (and is independent of iron acquisition) for growth in human urine in contrast to the E. coli ABU prototype 83972 isolate. Our result of S. agalactiae being independent of iron for optimal growth in urine is consistent with another study conducted with S. agalactiae reference strain A909 (555). In that study S. agalactiae was tested in iron-restricted media (THB treated with a divalent cation chelators, nitrilotriacetic acid) and the results showed that growth was independent of iron.

It is also important to discuss the potential limitations of our iron limiting experiments, such as species difference between E.coli and S. agalactiae in utilizing iron as a growth supplement. For example, it is possible that 200µM 2,2’-dipyridyl may not be sufficient
to create an iron restricted media for *S. agalactiae* even though this concentration is sufficient to create iron limited media for ABU *E. coli* 83972. Literature suggests for other bacterial species like *S. pyogenes* requires tenfold higher concentration of iron chelators such as 2,2'-dipyridyl or nitrilotriacetic acid to inhibit the growth of the bacteria (564) compared to the concentration required to inhibit the growth of *E. coli*. Therefore, more optimization may be required to determine the concentration of 2,2'-dipyridyl which can provide an iron restricted media but is not a bactericidal. This would be useful in the future, to further study iron dependency of different *S. agalactiae* isolates.

In this study we also investigated the virulence genes that might help *S. agalactiae* to survive and multiply in high numbers in urine and cause ABU or acute UTI. Virulence genes including those for siderophores were investigated in this context. The role of iron in biofilm formation has been recognized in different uropathogenic bacterial species such as *E. coli* (UTI strain VR50) (544) and *P. aeruginosa* (565). While we did not investigate biofilm formation in this study, we did determine the presence of genes that may be relevant to this phenotype. We screened ABSA 834 for thirteen virulence genes including *fhuD*, *OppA1-F* and *guaA*. In our PCR screening we found that *fhuD* is present in *S. agalactiae* COH1, A909 and ABSA 834. The *fhuCBG* operon was identified in *S. aureus* and all three genes showed sequence similarities to *fhu* genes in *B. subtilis*, where *fhu* stands for ferric hydroxamate uptake (566). Later studies identified *fhuD1* and *fhuD2* in *S. aureus*, which are involved in transport of iron(III) hydroxamate complexes (567). In *S. agalactiae*, *fhuD* was sequenced and identified in a prior study and was shown to be similar to *fhuD1* and *fhuD2* of *S. aureus* (555). In studies showing the presence of *fhuD* in nine clinical *S. agalactiae* isolates the FhuD protein displayed higher affinity for iron(III)-desferroxamine compared to an equivalent iron-binding protein in *S. aureus* (555, 568). However, the findings of Clancy *et al.*, were inconsistent with our observation on *S. agalactiae* isolates; they showed that *S.
*agalactiae* growth were inhibited in iron depleted media with 20mM nitriloacetic acid (555). This indicates that this organism can be dependent on iron for growth under some conditions and further optimization of the assays reported here are required.

*S. agalactiae* requires different amino acids for growth. The requirement of amino acids by *S. agalactiae* is usually satisfied via the uptake of peptides (557). In many microorganisms uptake of peptides such as oligopeptides is mediated through binding-protein dependent-permeases that belongs to ATP-binding cassette (ABC) transporters (569). For example, *L. lactis* possess two ABC transporters such as DppA-E and OppA-F for di- and oligopeptides respectively (570-573). In group A streptococcus (GAS) transport of dipeptides is carried out by Dpp whereas hexapeptides uptake is carried out by Opp (574). Uptake of dipeptides in *S. agalactiae* involves both ABC transporters DppA-E and OppA1-F and the proton driven symporter DpsA (557). A study conducted by Jones A. L. *et al.*, using signature-tagged transposon mutagenesis (STM) identified the di-peptide ABC transporter gene *dppA-E* in *S. agalactiae* and BLAST analysis of *dppA* showed 93% identity to GAS *dppA* (552). Oligopeptide uptake in *S. agalactiae* is mainly mediated by ABC oligopeptide transporters OppA1-F (557). These di-peptide and oligopeptide permeases not only provide bacteria with required amino acids but are also associated with virulence. For example, in GAS transcription of the cysteine protease *speB*, which is a known virulence gene, is positively regulated by Opp and Dpp (574, 575). The oligopeptide permeases OppA1-F of *S. agalactiae* were shown to stimulate the adherence of bacteria to epithelial cells and modulate binding to fibrinogen, and fibronectin. The study also showed that OppA1-F controls expression of *fsbA*, which encodes a fibrinogen-binding adhesin (557). Considering all these prior studies, it would appear that ABC transporter genes are involved in peptide uptake in *S. agalactiae* and may be directly linked with the regulation of virulence factors. Based on this insight, we investigated the presence of these genes in ABSA
In our PCR screening we found that all the thirteen genes including dppA-B and oppA1-F were present ABSA 834. These genes were also present in S. agalactiae COH1, and A909. Future studies will be needed to investigate the presence of these genes in a larger collection of ABSA and UPSA isolates to determine if there is any correlation between gene presence or absence and urovirulence or an ability to grow in urine. Based on our data in this Chapter it can be stated that testing of more S. agalactiae strains would be valuable to study whether these bacteria are able to utilize these peptide permeases systems for growth, survival and virulence.

Another interesting gene which may have a role in supporting growth in urine of S. agalactiae by offering biosynthesis of purines for metabolism (558) and resistance to mildly acidic conditions (pH 5.0-5.5) is guaA (548). This gene was found to be present in ABSA 834 and in the two reference strains tested, and encodes GMP synthase. guaA is separated from the upstream guaB gene by a 68bp intercistronic region in the polycistronic guaBA operon in E. coli K12 (546). Studies in E. coli and S. agalactiae have shown that guaA is involved in guanine (one of the purines) biosynthesis (270, 552). De novo purine biosynthesis is known to be associated with growth, virulence and infections of many bacteria (552, 576). guaA was identified in S. agalactiae A909 using signature-tagged mutagenesis to identify genes involved in the growth and survival of this organism in the host (552). Reference strain S. agalactiae A909 has also been reported to be prototrophic for purine biosynthesis (558). The presence of guanine and its derivatives in human urine has been reported by several studies (334, 550, 551). One study analyzed urine samples of twenty healthy volunteers using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and found a total of 8-hydroxylated guanine species at a level of 212nmol/24h. They reported that oxidized guanine base represented 64% of the guanine present (549). The synthesis of guanine has been identified as a requirement for uropathogenic E. coli
for growth in human urine (384). Russo et al., showed that a *E. coli* guaA::TnphoA′4 mutant was unable to grow in human urine *in vitro* and was unable to infect the mouse urinary tract with significantly lower virulence in the *in vivo* mouse model (270).

As prior noted, resistance to mildly acidic conditions may be important for survival and or growth of *S. agalactiae* in human urine, and there is evidence that *guaA* is involved in acid stress responses. Considering urine is mildly acidic it is important to note that a *L. lactis* *guaA* transposon insertional mutant showed many fold increased acid tolerance compared to the WT (548). Their study identified four genes including *guaA* that had significant effects on acid tolerance where the mutant exhibited higher tolerance and improved survival under oxidative stress and heat shock compared to the WT (547, 548). Based on their results, the authors concluded that an alteration of the guanine nucleotides biosynthesis flux prior to a lethal acid challenge leads to improved bacterial survival (548). Taken together, these data indicate that guanine usage and biosynthesis in bacteria can have major and sometimes unexpected influence (such as improved survival/higher resistance to acid) on survival in difference conditions. Our PCR screening and detection of *guaA* in ABSA 834 is important, considering *guaA* may play different roles in supporting growth of *S. agalactiae* in acidic human urine which contain purines (including guanine). Finally, the optimized PCR conditions developed in this Chapter will be useful for screening of other ABSA and UPSA isolates in future studies.

The main question for this Chapter was how *S. agalactiae* survive and grow in human urine, and importantly, how this phenotype can be measured accurately. Our data has shown that both turbidity and colony count measures are important to fully understand the growth patterns of difference *S. agalactiae* isolates. Through experimentation, we
have also answered several key questions in this regard. It appears that unlike *E. coli*, *S. agalactiae* does not use iron for urine growth but does use guanine. The presence of multiple virulence genes related to metabolic activity in ABSA 834 will be an area for future functional studies. The phenotypes identified in this Chapter, particularly related to guanine, may impact colonization of patients and contribute to high grade ABU (>10⁴ CFU/ml). Future studies could address this, such as by using a mouse model for infection assays *in vivo*. Future assays focusing on the factors present in human urine that *S. agalactiae* utilizes for growth will help to define the survival strategies of this organism in the human urinary tract and how this relates to the clinical conditions of *S. agalactiae* UTI and ABU.
Chapter 8

General discussion

Bacteriuria is one of the most frequent infectious diseases and a massive financial burden on healthcare costs estimated at US$2.5-3.5 billion in medical expenses and societal costs annually in the USA alone (209, 210). Bacterial UTI due to Gram-positive pathogens occurs frequently and represents a major health problem in Australia and world-wide. The capacity of microorganisms for urine growth may aid in establishing long-term bacteriuria and is relevant to many causal species including S. agalactiae. However, to date, there is virtually no information on the mechanisms of UTI caused by Gram-positive uropathogens such as S. agalactiae and little research has been performed on the ability of this organism to grow in human urine. In terms of investigating bacterial growth in urine, the continued use of experimental models such as SHU medium for human urine, and UTI animal models of human disease will drive new discoveries in our understanding of how bacteriuria progresses in the host.

One of the key aims of this thesis was to investigate the ability of different S. agalactiae strains, derived from distinct clinical backgrounds, to grow in human urine. The hypothesis was that different growth abilities may be used by the bacterium to persist in the niche of the host urinary tract or bladder and thus contribute to the pathogenesis of UTI. Specifically, this study investigated the diversity in the ability of ABSA and UPSA to grow in human urine in vitro. Initially, the study assessed the utility and reliability of optical density (OD600nm) and colony count approaches for measuring S. agalactiae growth in urine. This work identified variables that affect the differences in optical density readings of different S. agalactiae isolates when grown in human urine, and defined the serotypes of S. agalactiae isolates selected through growth phenotypes in human urine based on different UTI clinical history. This study also identified the correlation between the ability to grow in human urine and the clinical
presentation/disease severity in patients with UTI. The genetic basis for uropathogenicity of *S. agalactiae* by whole genome sequencing in a defined collection of UTI isolates was also investigated. Finally, the study focused on the virulence of representative *S. agalactiae* isolates from different patient groups for colonization studies in a mouse UTI model, which compared the bladder colonization ability of ABSA and UPSA. In this regard, experiments were conducted to assess the contribution of selected virulence genes such as *meaE* and *guaA* of *S. agalactiae* in utilization of human urine as a growth medium and the potential role in pathogenicity. Overall therefore, this thesis applied growth assays in human urine, serotyping, metabolic profiling, genome sequencing, targeted gene deletion, *in vitro* growth assays in human urine and *in vivo* assays in UTI mouse models to address the question of *S. agalactiae* uropathogenesis.

Our understanding of the ability of *S. agalactiae* to grow in human urine and bacterial genes or constituents of urine which may support the growth and survival of *S. agalactiae* in the urinary tract or bladder is very limited. Through initial growth assays in human urine with ABSA and UPSA isolates, this study pinpointed that *S. agalactiae* isolates from different patient populations (ABSA and UPSA isolates) have a diverse ability of growth fitness in human urine. Serotyping of a selected forty fast growers in human urine revealed that type III (35%) serotypes were predominant which was consistent with the findings of another study conducted in United States (16). Metabolic profiling of a representative ABSA isolate, 1014, and UPSA isolate 807, demonstrated efficient utilization of D, L-Malic acid and L-Malic acid in the former which was shown to be directly related to functional expression of the malic enzyme (ME) metabolic pathway (289). Interrogation of the draft assembled genomes of ABSA 1014 and UPSA 807 highlighted a frameshift mutation in a poly A sequence at the 5’ end of *maeK* of UPSA 807; this mutation, we hypothesized would lead to the production of a truncated non-functional protein. Subsequent growth assays comparing a ME-deficient ABSA
834ΔmaeE mutant to the wild-type (WT) ABSA 834 strain in pooled NHU and SHU, both containing 40mM malic acid, revealed that the ABSA 834ΔmaeE mutant was significantly attenuated for growth in both NHU and SHU compared to the WT ABSA 834 strain. Thus, these data collectively show that a) there are differences in the abilities of UPSA and ABSA to grow in human urine, b) serotype distributions of fast growers are frequently serotype III S. agalactiae, and c) the ME metabolic pathway is involved in S. agalactiae growth in urine.

In this project, mouse in vivo assays and bio-plex analysis provided insight into host immune responses and differences in bacterial survival in the mouse urinary tract between ABSA and UPSA isolates. Comparison of the ability of ABSA and UPSA to establish bladder colonization in the frequently used mouse model based on female C57BL/6 mice revealed that there was significantly higher numbers of bacteria recovered from bladders and urine of mice infected with ABSA 1014 compared to UPSA 807 at 24h post infection. The effect of an ABSA maeE mutation (encoding the malate oxidoreductase used for malic acid metabolism) on mouse colonization showed that maeE played a critical role in bladder colonization as the maeE mutant showed a significantly reduced bladder colonization load compared to WT ABSA. The differential immune and inflammatory response of the mouse bladder to ABSA and UPSA showed diverse patterns of immune responses. These responses included a) unchanged cytokine production compared to controls, b) up regulated cytokines levels compared to controls, c) different amount of cytokine production in bladders between ABSA and UPSA infected, and d) different cytokine patterns between WT ABSA 834 and its maeE-deficient mutant in infected bladders.

In Chapter 7, we investigated whether there is any correlation between turbidity and CFU colony counts in cultures of S. agalactiae in human urine, whether S. agalactiae is dependent on iron for growth in human urine, assessed for the presence of thirteen virulence genes including fhuD (iron-related metabolism) and guaA (guanine-related
metabolism) in ABSA 834, and the role of guaA in supporting the growth of ABSA 834 in mildly acidic human urine. The results confirmed that both colony count and turbidity measurements are important to accurately gauge the growth of these chain-like structured bacteria in human urine. The PCR results were interesting because these revealed the presence of all thirteen genes in ABSA 834 along with reference strains COH1 and A909. These data suggest that these virulence genes are widely distributed among S. agalactiae strains including ABSA type organisms; however, at present there is no information on the expression of these genes and whether these are regulated differently in different strains; analysis of differential gene expression in the different types of UPSA and ABSA would be a good area for future investigation. In the process of these experiments, we were also able to show one gene, guaA plays an important role in urine growth of S. agalactiae, although this data must be treated as preliminary and needs assays with a complemented mutant to assess restoration of the phenotype. Finally, the results of growth experiments in human urine using ABSA 834 and investigating the dependence on iron suggest that iron availability in human urine does not influence the growth of S. agalactiae but does support the growth of ABU E. coli.

In conclusion, the current study identified for the first time that S. agalactiae can grow in human urine and metabolize different constituents present in human urine. Future studies using transcriptional profiling will now be of interest to expand the knowledge of different gene activation pathways during ABSA growth in urine beyond malic acid or guanine metabolism. The metabolome finding, based on ABSA 1014 and UPSA 807 highlights the importance of utilization of malic acid for urine growth, which will be of interest to now study in other bacterial species that cause UTI. Analysis of functionally intact ME metabolic pathway in other ABSA, and the need for this for robust growth in human urine will also be of interest for future work. In our PCR screening we found that all the selected thirteen genes including dppA-B and oppA1-F were present ABSA 834.
Future studies will be needed to investigate the presence of these genes in a larger collection of ABSA and UPSA isolates and to analyse the expression of these genes in different isolates to determine if there is any correlation between gene presence or expression and urovirulence or an ability to grow in urine. Optimized PCR conditions developed in Chapter 7 will be useful to perform such further studies. The phenotypes identified in Chapter 5 and 7, particularly related to malic acid and guanine metabolism, may impact colonization of patients and contribute to high grade ABU (>104 CFU/ml). Future studies could address this, such as by using a mouse model for infection assays in vivo. Liquid chromatography on collected pooled human urine which used for growth assays would be a choice to analyse how elevated levels of urine components in each batch of human urine impacts on the growth pattern of S. agalactiae. For example, changes in growth phenotypes of S. agalactiae in different batches of human urine could reflect different urine constituents between batches. Such comparison could help to identify different carbon, nitrogen or other components present in human urine which serve as an energy source for growth of S. agalactiae in urine. Microbiologically, we need to define the lifestyle adaptations, other than those described for D-serine, malic acid, guanine and iron acquisition that microbes use to aid bacteruric potential, and define the molecular basis of urine growth in ABSA organisms. Such future assays focusing on the other factors present in human urine that S. agalactiae utilizes for growth will help to define the complete repertoire of survival strategies that this organism uses in the human urinary tract and how this relates to the clinical conditions of S. agalactiae UTI and ABU.
Appendix 1

Supplementary Data of Chapter 2 Publication

Table S1. ABU prevalence, risk factors and comorbidities in distinct patient populations shown alongside specific prevalence rates of causal microbes.
<table>
<thead>
<tr>
<th>Patient Population</th>
<th>ABU Prevalence (%), Risk Factors, and Comorbidities</th>
<th>References (relate to left column)</th>
<th>Causal Microbe Prevalence in Given Patient Population (%)</th>
<th>References (relate to left column)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Adult Women</td>
<td>1.0-5.0 / 2.8-8.6</td>
<td>(173, 177, 577-581)</td>
<td>E. coli 38.4-91.0</td>
<td>(580, 582, 583)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Enterococcus 4.0-33.2</td>
<td>(580)</td>
</tr>
<tr>
<td>Premenopausal (&lt;50 y) / Postmenopausal (50-70 y)</td>
<td></td>
<td></td>
<td>K. pneumonia 6.1</td>
<td>(16, 34, 39, 43, 580)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>S. agalactiae 1.1-5.5</td>
<td>(580)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Serratia 3.8-5.1</td>
<td>(585)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S. aureus 0.1-2.2</td>
<td>(580)</td>
</tr>
<tr>
<td>Healthy Adult Men</td>
<td>0.8-2.0</td>
<td>(173, 177, 577, 578, 586-589)</td>
<td>E. coli 13.8-25.0</td>
<td>(586, 587)</td>
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<td></td>
<td>Prostatic hypertrophy, history of instrumentation of the urogenital tract or urogenital tract surgery, anatomic or functional urinary tract abnormalities, prostatic infection</td>
<td></td>
<td>Enterococcus 22.5</td>
<td>(586)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pseudomonas 11.6</td>
<td>(587)</td>
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<td></td>
<td>Klebsiella 7.7</td>
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<td></td>
<td></td>
<td></td>
<td>Staphylococcus 2.0-6.9</td>
<td>(587, 590)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Streptococcus 5.4</td>
<td>(587)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Serratia 2.0</td>
<td>(587)</td>
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<td>Pregnant Women</td>
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<td>(177, 184, 577, 579, 591-597)</td>
<td>E. coli 27.1-86.0</td>
<td>(171, 594, 597-600)</td>
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<tr>
<td></td>
<td>Socioeconomic status, diabetes, sickle cell disease and trait, multiparity, history of UTI, and anatomic or functional urinary tract</td>
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<td>Enterococcus 4.0-15.8</td>
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<td>Klebsiella 4.0-15.8</td>
<td>(171, 184)</td>
</tr>
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<td></td>
<td>Serratia 2.0</td>
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<td>Staphylococcus</td>
<td>G. vaginalis, U. ureolyticum, S. agalactiae</td>
<td>Pseudomonas, Providencia</td>
<td>Enterococcus</td>
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<td>1.0-11.3²</td>
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<td>(599)</td>
<td>(592, 594)</td>
<td>(594, 597)</td>
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<td>Diabetic Adults</td>
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<tr>
<td>Women / Men³</td>
<td>6.1-30.0 / 0.7-10.1</td>
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<td>♀ / 29.4</td>
<td>(196, 422, 606, 607, 609, 610, 617, 618, 620)</td>
<td>(422, 606, 607, 617, 618)</td>
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<tr>
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<td></td>
<td></td>
<td>(422, 609, 620)</td>
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<td></td>
<td>Klebsiella</td>
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<tr>
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<td></td>
<td>(382, 422, 606, 609, 610, 618)*</td>
<td>(582, 617, 618)</td>
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<td></td>
<td></td>
<td>(422, 609)</td>
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<tr>
<td></td>
<td>Streptococcus</td>
<td>4.3.0-12.0</td>
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<tr>
<td></td>
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<td>(606, 607, 609, 610, 617, 620)</td>
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(Consensus data for the above)
remains unclear since multiple independent studies have reported conflicting results)

<table>
<thead>
<tr>
<th>Community Dwelling Elderly Adults (&gt;70 y)</th>
<th>Women / Men³</th>
<th>Neurological disease causing reduced bladder motility or continence, diabetes mellitus, primary biliary cirrhosis, reduced mobility, structural abnormalities, indwelling urinary catheter, oestrogen treatment in women, high postvoid residual (PVR) volume in men, usage of urethral condom catheter in men, history of UTI</th>
<th>(169, 316, 577, 588, 589, 623-633)</th>
<th>ABU involves renal infection in &gt;50% of cases according to: (634, 635)</th>
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<td>(588, 589, 623, 635)</td>
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</tbody>
</table>

E. aerogenes 4.0-4.3 ♀

G. vaginalis / 11.8 ♂

Enterococcus

1.2-1.4 ♀ / 35.3 ♂

P. mirabilis 4.0 ♀

Citrobacter freundii
<table>
<thead>
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<th>Institutionized Adults and Elderly Women / Men</th>
<th>Patients with Indwelling Catheter</th>
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</thead>
<tbody>
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<td></td>
<td>25.0-75.0 / 14.0-52.0 Neurological disease causing reduced bladder motility or continence, functional impairment, reduced bowel continence, usage of urethral condom catheter in men</td>
<td>9.0-23.0 / 100 (584, 653-660)</td>
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<td>Staphylococcus</td>
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<td>12.0-35.0 (654, 658, 661)</td>
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<td>CMP&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.0-10.3</td>
<td>4.0-6.8 (654, 661)</td>
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<td>E. coli</td>
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<td>5.0-10.2 (654, 661)</td>
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<td>2.9-24.0 (661)</td>
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<td>P. mirabilis</td>
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<td>8.4-21.0 (661)</td>
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<td>K. pneumonia</td>
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</tr>
<tr>
<td>E. faecalis</td>
<td>4.0-16.2 (661)</td>
<td></td>
</tr>
<tr>
<td>Providencia</td>
<td>3.0-10.3</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus</td>
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<tr>
<td>M. morganii</td>
<td>12.0-35.0 (654, 658, 661)</td>
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<tr>
<td>M. morganii</td>
<td>19.0-25.0 (661)</td>
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<tr>
<td>Enterobacter, Klebsiella</td>
<td>8.4-21.0 (661)</td>
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</tr>
<tr>
<td>S. aureus</td>
<td>2.9-5.7</td>
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</tr>
<tr>
<td>S. agalactiae</td>
<td>2.0-4.3</td>
<td></td>
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</tbody>
</table>

1Relates to Population; 2Pregnant women (169, 171, 177, 592), men undergoing urologic interventions, patients with abnormal urinary tracts, or persistent bacteriuria 48h after intermittent catheterization, or instrumentation with probability of bleeding (169, 639, 662) recommended for therapy; prevalence 26% for S. agalactiae in diabetic gravidas (382); 3If possible, prevalence shown by gender: female ♀, male ♂; mixed infections excluded; 4CMP, Citrobacter, Morganella, Providencia.
Appendix 2

Supplementary Data of Chapter 4

Growth experiments of 358 Isolates in Human Urine: Among 358 S. agalactiae isolates tested for ability to grow in human urine 40 ‘fast’ and 25 ‘slow’ growers were identified with the aid of both OD600nm and colony counts measurements. Colony counts of ten selected fast growers and slow growers are demonstrated in Fig 1 and Fig 2 of this chapter. Additional colony count data figures of rest of the selected fast growers (30 isolates) and selected slow growers (15 isolates) are demonstrated in figure S1a-S1c and fig S2a-S2b respectively.

Fig S1a-S1c: Colony counts of (10 isolates per graph) fast growers in human urine.
Fig S1b: Colony Count of Fast Growers in Human Urine

Fig S1c: Colony Count of Fast Growers in Human Urine
Fig S2a-S2b: Colony counts of the additional 15 slow growers of the selected 25 isolates in human urine.
**Fig S3a-S3c:** Absorbance reading at OD600nm of additional fast growers (30 isolates of selected 40 fast growers) in human urine. Ten isolates per graph.

**Fig S3a: OD600 of Fast Growers in Human Urine**

**Fig S3b: OD600 of Fast Growers in Human Urine**
Fig S3c: OD600 of Fast Growers in Human Urine
**Fig S4a-S4b:** Absorbance reading at OD600nm of the additional slow growers (15 isolates of selected 25) in human urine slow growing isolates.
Fig S5a-S5f: Supplementary figures of colony count data of acute UTI or cystitis group (Group 1) grew in human urine (0h-72h).

**Fig S5a: Colony Count of Acute UTI Group in Human Urine**

![Graph showing colony count data for different isolates](image1)

**Fig S5b: Colony Count of Acute UTI Group in Human Urine**

![Graph showing colony count data for different isolates](image2)
Fig S5e: Colony Count of Acute UTI Group in Human Urine

Fig S5f: Colony Count of Acute UTI Group in Human Urine
Fig S6a-S6i: Supplementary figures of colony count data of low-grade ABU group (Group 2) grew in human urine (0h-72h).
Fig S6e: Colony Count of Low-Grade ABU Isolates in Human Urine

Fig S6f: Colony Count of Low-Grade ABU Isolates in Human Urine
Fig S6i: Colony Count of Low-Grade ABU Isolates in Human Urine

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Time (h)</th>
<th>Number of bacteria (Log_{10} CFU/ml)</th>
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<tbody>
<tr>
<td>Isolate 1007</td>
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</tr>
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<td>Isolate 1021</td>
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<tr>
<td>Isolate 1024</td>
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<td>Isolate 1053</td>
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<td></td>
</tr>
<tr>
<td>Isolate 1062</td>
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</tbody>
</table>
Fig S7a-S7u: Supplementary figures of colony count data of high-grade ABU group (Group 3) grew in human urine (0h-72h).
Fig S7b: Colony Count of High-Grade ABU Isolates in Human Urine

Fig S7c: Colony Count of High-Grade ABU Isolates in Human Urine
Fig S7d: Colony Count of High-Grade ABU Isolates in Human Urine

Number of bacteria (Log_{10} CFU/ml)

Time (h) 0 6 12 24 36 48 60 72

Fig S7e: Colony Count of High-Grade ABU Isolates in Human Urine

Number of bacteria (Log_{10} CFU/ml)

Time (h) 0 6 12 24 36 48 60 72
Fig S7h: Colony Count of High-Grade ABU Isolates in Human Urine

Fig S7i: Colony Count of High-Grade ABU Isolates in Human Urine
Fig S7j: Colony Count of High-Grade ABU Isolates in Human Urine

Fig S7k: Colony Count of High-Grade ABU Isolates in Human Urine
Fig S7l: Colony Count of High-Grade ABU Isolates in Human Urine

Fig S7m: Colony Count of High-Grade ABU Isolates in Human Urine
Fig S7n: Colony Count of High-Grade ABU Isolates in Human Urine

Fig S7o: Colony Count of High-Grade ABU Isolates in Human Urine
Fig S7p: Colony Count of High-Grade ABU Isolates in Human Urine

Fig S7q: Colony Count of High-Grade ABU Isolates in Human Urine
Fig S7r: Colony Count of High-Grade ABU Isolates in Human Urine

Fig S7s: Colony Count of High-Grade ABU Isolates in Human Urine
Fig S7t: Colony Count of High-Grade ABU Isolates in Human Urine

Fig S7u: Colony Count of High-Grade ABU Isolates in Human Urine
**Fig S8a-S8f**: Supplementary figures of absorbance reading at OD600nm of acute UTI or cystitis group (Group 1) grew in THB (0h-72h).

**Fig S8a: OD600nm of Acute UTI Group in THB**

**Fig S8b: OD600nm of Acute UTI Group in THB**
**Fig S9a-S9i**: Supplementary figures of absorbance reading at OD600nm of low-grade ABU group (Group 2) grew in THB (0h-72h).

**Fig S9a**: OD600nm of Low-Grade UTI Group in THB

**Fig S9b**: OD600nm of Low-Grade UTI Group in THB
Fig S9c: OD600nm of Low-Grade UTI Group in THB

Fig S9d: OD600nm of Low-Grade UTI Group in THB
Fig S9g: OD600nm of Low-Grade UTI Group in THB

Fig S9h: OD600nm of Low-Grade UTI Group in THB
Fig S9i: OD600nm of Low-Grade UTI Group in THB

Absorbance at 600nm

Time (h)
Fig S10a-S10u: Supplementary figures of absorbance reading at OD600nm of high-grade ABU group (Group 3) grew in THB (0h-72h).

**Fig S10a: OD600nm of High-Grade UTI Group in THB**

- Isolate 2
- Isolate 7
- Isolate 13
- Isolate 14
- Isolate 16
- Isolate 17
- Isolate 18
- Isolate 20
- Isolate 24
- Isolate 30

**Fig S10b: OD600nm of High-Grade UTI Group in THB**

- Isolate 32
- Isolate 35
- Isolate 40
- Isolate 49
- Isolate 53
- Isolate 55
- Isolate 56
- Isolate 72
- Isolate 76
- Isolate 83
Fig S10m: OD600nm of High-Grade UTI Group in THB

Fig S10n: OD600nm of High-Grade UTI Group in THB
Fig S10s: OD600nm of High-Grade UTI Group in THB

Absorbance at 600nm

Time (h)

Fig S10t: OD600nm of High-Grade UTI Group in THB

Absorbance at 600nm

Time (h)
Fig S10u: OD600nm of High-Grade UTI Group in THB
Appendix 3

Supplementary Data of Chapter 6

Fig S1a – S1o: Immune response data of additional 15 cytokines and chemokines out of 23 tested against ABSA 1014 (▼) and UPSA 807 (♦) isolates compared to ctrl group (●). Data also includes the comparison of immune response between ABSA 834 (■) and ABSA 834∆maeE (▲). Data points represents twenty mice in each infected or control group from two independent experiments with median calculated. Statistical analyses were performed using GraphPad Prism v5 and SPSS v22.0.
Fig S1j: GM-CSF

Fig S1k: IFN-γ
Fig S1l: KC

Fig S1m: MCP-1
Fig S1n: RANTES

Fig S1o: TNF-α
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