Gram-Positive Uropathogens, Polymicrobial Urinary Tract Infection, and the Emerging Microbiota of the Urinary Tract

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Abstract

Gram-positive bacteria are a common cause of urinary tract infection (UTI), particularly among individuals who are elderly, pregnant, or who have other risk factors for UTI. Here we review the epidemiology, virulence mechanisms, and host response to the most frequently isolated Gram-positive uropathogens: *Staphylococcus saprophyticus, Enterococcus faecalis*, and *Streptococcus agalactiae*. We also review several emerging, rare, misclassified, and otherwise underreported Gram-positive pathogens of the urinary tract including *Aerococcus, Corynebacterium, Actinobaculum,* and *Gardnerella*. The literature strongly suggests that urologic diseases involving Gram-positive bacteria may be easily overlooked due to limited culture-based assays typically utilized for urine in hospital microbiology laboratories. Some UTIs are polymicrobial in nature, often involving one or more Gram-positive bacteria. We herein review the risk factors and recent evidence for mechanisms of bacterial synergy in experimental models of polymicrobial UTI. Recent experimental data has demonstrated that, despite being cleared quickly from the bladder, some Gram-positive bacteria can impact pathogenic outcomes of co-infecting organisms. When taken together, the available evidence argues that Gram-positive bacteria are important uropathogens in their own right, but that some can be easily overlooked because they are missed by routine diagnostic methods. Finally, a growing body of evidence demonstrates that a surprising variety of fastidious Gram-positive bacteria may either reside in or be regularly exposed to the urinary tract and further suggests that their presence is widespread among women, as well as men. Experimental studies in this area are needed; however, there is a growing appreciation that the composition of bacteria found in the bladder could be a potentially important determinant in urologic disease, including susceptibility to UTI.

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Gram-Positive and Polymicrobial UTI Epidemiology

Uncomplicated UTI

Uncomplicated urinary tract infection (**UTI**) is most common in young, sexually active, nonpregnant, premenopausal women. Gram-negative bacteria are isolated from 75 to 95% of these infections [1]. The remaining proportions of uncomplicated UTI are associated with a variety of organisms, including the Gram-positive bacteria *Staphylococcus saprophyticus, Enterococcus faecalis, Streptococcus agalactiae* (group B *Streptococcus*, **GBS**), and other less frequently isolated organisms. In demographic groups such as in pregnant women and the elderly, Gram-positive bacteria are found more often as etiologic agents of UTI. Symptoms associated with uncomplicated UTI caused by Gram-positive uropathogens are similar to those caused by Gram-negative organisms and usually include dysuria, urinary frequency, urinary urgency, and/or suprapubic pain. Fever, chills, costovertebral-angle tenderness, flank pain, and/or nausea are suggestive of upper urinary tract (kidney) involvement.

Point of care diagnosis of UTI

While the gold standard for UTI diagnosis is bacterial culture of the urine, dipstick urinalysis is commonly used in point-of-care diagnosis. In some clinical settings such as with infants, leukocyte esterase (LE) and pyuria (by dipstick analysis) have a very high sensitivity and specificity for UTI (>90% as defined by the culture of a uropathogen from urine with >100,000 colony forming units (CFU) per ml) [2, 3]. However, in contexts such as pregnancy, dipstick analysis using LE, pyuria, or presence of nitrites is less reliable as an indication of UTI per the microbiological definition of 10^5 CFU/ml cutoff [4, 5]. While dipstick urinalysis that is positive for LE and/or nitrites in a clean-catch urine sample is consistent with a UTI diagnosis, these tests can miss UTIs that meet the gold standard of bacteriuria diagnosis in relation to adverse outcomes in pregnancy (e.g. the microbiological 10^5 CFU/ml definition). One likely explanation is that nitrite tests are likely to be negative if the infecting organism does not reduce nitrate, as is the case for most Gram-positive uropathogens including *S. saprophyticus,* enterococci, and group B *Streptococcus* [6, 7]. Given the higher prevalence of Gram-positive bacteria as causes of UTI in certain populations such as the elderly, it is perhaps not surprising that some studies conclude that LE and nitrite are inadequate for UTI screening in this setting [8]. In short, while dipstick urinalyses can help to quickly identify UTI caused by Gram-negative bacteria, they are less useful for infections involving Gram-positive uropathogens and perform poorly in ruling out these infections with certainty.

Complicated UTI

Complicated UTI is defined as cystitis or pyelonephritis that occurs in individuals with predisposing anatomic, metabolic, or functional risk factors that make UTI more difficult to treat. Complicated UTIs often occur in nosocomial and/or institutional settings, particularly in individuals with structural or functional alterations of the urinary tract (often associated with urinary catheterization), or other underlying renal, metabolic, or immunological disorders [9]; these populations are at greater risk of Gram-positive and polymicrobial UTI [10, 11]. Another less frequently recognized anatomic risk factor for UTI is female genital

cutting (FGM). A recent meta-analysis of five comparative studies showed that women who had experienced FGM were at 3-times higher risk of UTI compared to those who were uncut [12].

Catheter associated UTI

Catheter-associated UTI (**CAUTI**) account for 40% of all nosocomial infections [13] and are the most common complication of indwelling urinary catheters [14, 15]. Catheter-associated bacteria are thought to be derived largely from the patient's own gut microbiota [16]. Bacteriuria occurs in 3–10% of patients following urinary catheterization [13, 17]. Catheter-associated bacteriuria is often asymptomatic [15] and there is no good way to distinguish between pathogenic CAUTI and asymptomatic bacteriuria (**ASB**); even the presence of neutrophils in the urine (pyuria), which are a strong identifier of uncomplicated UTI, is not a good diagnostic indicator of CAUTI [13]. Catheter-associated bacteria are largely in a biofilm state and are thus recalcitrant to antibiotic treatment [13, 18, 19]. However, if left untreated, these infections can lead to severe complications such as acute pyelonephritis, bacteremia, urosepsis, and death [13, 16]. While the well-adapted uropathogenic *E. coli* cause the majority of non-catheter-associated UTI in the community, the diversity of species associated with CAUTI is greater. For example, enterococci are rarely associated with community-acquired UTI but play a prominent role in the pathogenesis of CAUTI and are among the predominant pathogens isolated from polymicrobial communities on the surface of indwelling urinary catheters and biliary stents [20–22].

Laboratory models to study Gram-Positive UTI

Model systems to recapitulate and study infection by Gram-positive uropathogens have been adapted from those used to study UTI caused by Gram-negative bacteria. Transurethral inoculation with 1.5 ml of *S. saprophtyicus* at 1×10^9 colony forming units (**CFU**)/ml into the bladders of female albino WISTAR rats showed that at 7 days post infection, both bladders and kidneys were colonized at similar levels, leukocytes were present in the urine, and bladder inflammation and epithelial damage noted [23]. Subsequent studies in which 50μl of *S. saprophyticus* at 5×10^7 CFU/ml was transurethrally inoculated into 7–8 week old C3H/HeN female mice showed significantly higher CFU in the kidney compared to the bladder at 6 hours post infection and 2, 7, and 14 days post infection [24]. Bacterial persistence in the kidneys was observed in C3H/HeN mice but not in C57BL/6 mice, indicating that host factors contribute to the ability of *S. saprophyticus* to cause UTI. Under the same infection conditions, GBS showed similar kidney tropism at 1, 7, and 14 days post infection [25]. Similarly, *E. faecalis* preferentially infects the kidneys of C57Bl/6, outbred Harlan Sprague Dawley, and BALB/c female mice; however, these models require a 200μl inoculum volume to consistently establish infection [26–28]. Since *E. faecalis* is more commonly associated with CAUTI than ascending UTI, foreign body-associated UTI models have been developed in mice and rats to mimic the conditions of patients with indwelling urinary catheters [29, 30]. In the murine model, catheter material is inserted transurethrally into the murine bladder prior to bacterial inoculation, where it remains throughout the course of infection. *E. faecalis* establishes a robust infection in the catheter-containing bladder, in the kidneys, and on the catheter material itself where it forms a biofilm that facilitates

persistent infection in the face of robust catheter-driven inflammation [30, 31]. The CAUTI murine model has recently been used to test the efficacy of novel UTI therapeutics and will continue to be useful in the search for antimicrobial agents aimed at preventing or dispersing Gram-positive biofilms that arise in catheterized individuals [32].

Epidemiology and animal models for polymicrobial UTI

The presence of multiple recognized uropathogens in midstream urine at titers >100,000 CFU/ml is consistent with a polymicrobial etiology of UTI. Polymicrobial infections occur most often among the elderly, immune compromised, and those with indwelling catheters, HIV, malignancy, and diabetes. Polymicrobial UTI is less common among young sexually active women. Since the highly polymicrobial microbiota of the GI and reproductive tracts are thought to be a major inoculation source leading to UTI, and since truly dual species or polymicrobial UTI do arise, several investigators have sought to examine the consequence of mixed microbial inoculation into the urinary tracts of model organisms. In a rat model, transurethral inoculation of *Staphylococcus saprophyticus* or *Proteus mirabilis*, resulted in ascending pyelonephritis significantly more often when the two organisms were inoculated together compared to single species infection, suggesting a synergistic virulence between the two species [33]. *P. mirabilis* also synergizes with UPEC in the murine urinary tract, such that co-infection gave rise to greater CFU for both *P. mirabilis* and UPEC, compared to either single species infection. The use of complementary, rather than competing, central metabolism pathways in the urinary tract by UPEC and *P. mirabilis* may limit competition and thus promote synergy between these two organisms [34]. Co-infection with the urease-positive Gram-negative organisms *P. mirabilis* and *Providencia stuartii* give rise to an increased incidence of urinary stones (urolithiasis) and bacteremia in a murine model of ascending UTI compared to monomicrobial infection [35], (ref), which may help explain why these organisms commonly co-occur in the urine of individuals with indwelling urinary catheters [20, 36, 37]. Similar studies in mice showed that *Pseudomonas aeruuginosa* and *E. faecalis* co-infection resulted in a more rapid development of pyelonephritis than observed when each species was inoculated alone [38]. Moreover, co-infection studies with group B *Streptococcus* and UPEC in a murine UTI model have demonstrated that the presence of GBS can modulate host immunity and alter host susceptibility to persistent high titer infection of the bladder and kidneys by UPEC [39]. Aged multiparous animals were particularly prone to UPEC infection in the context of GBS, demonstrating ~1000-fold higher titer UPEC infection in the presence of GBS compared to age-matched nulliparous controls [40]. Microscopic and microbial culture examinations, as well as culture-independent DNA sequence analysis of bacterial biofilms found on urinary catheters, show that CAUTI biofilms are often polymicrobial in nature [41–45]. By analogy to mixed species effects in ascending UTI, the nature of the mixed microbial community in CAUTI may also influence the spectrum or severity of sequelae.

In the next section of this chapter, we will examine the epidemiology, virulence mechanisms, and host response to the most frequently isolated Gram-positive uropathogens: *Staphylococcus saprophyticus, Enterococcus faecalis*, and *Streptococcus agalactiae*.

Urinary Tract Infection caused by Staphylococci

Epidemiology of *S. saprophyticus* UTI

Staphylococcus saprophyticus is a Gram-positive, coagulase negative, nonhemolytic coccus. Colonies of *Staphylococcus saprophyticus* are often yellow-pigmented [46]. *S. saprophyticus* causes 5–20% of community-acquired UTIs [47] and up to 42% of UTI among 16–25 year old women [48]. *S. saprophyticus* is second only to UPEC as the most common cause of uncomplicated UTI in this population [49, 50]. Similar to UPEC infection, recent sexual intercourse is also a risk factor for *S. saprophyticus* UTI [51, 52]. Over 40% of young, sexually active women are colonized with *S. saprophyticus* in the rectum, urethra, or cervix at any given time [52]. It is thought that a major source of urethral inoculation is the gastrointestinal (**GI**) microbiota. However, one study found no correlation between GI colonization and subsequent *S. saprophyticus* UTI [52]. *S. saprophyticus* colonization and UTI display an interesting seasonal variation, with the greatest prevalence in the late summer and early fall [48, 52–54]. The cause for this seasonality is not well understood. In the absence of complicating conditions, *S. saprophyticus* infection rarely causes UTI in males, but has been associated with urethritis and up to 17% of prostatitis [53, 55, 56]. One study of older men presenting with UTI in a veteran's hospital found that the presence of *S. saprophyticus* was very rare (3 of 9314 urine samples were *S. saprophyticus* positive) [57]. When it is observed, male UTI caused by *S. saprophyticus* is found predominantly in elderly or institutionalized individuals [58]. UTI symptoms caused by *S. saprophyticus* are similar in spectrum to those caused by *E. coli,* but can be more severe than in patients with *E. coli* UTI [59, 60]; approximately 40% of patients with *S. saprophyticus* UTI present with acute pyelonephritis [58, 61].

Novobiocin resistance is a laboratory hallmark for identification of *S. saprophyticus*. However, antibiotic resistance in *S. saprophyticus* is uncommon [62]. As is the case for many coagulase negative staphylococcus species (**CoNS**) [63], methicillin-resistance can occur in *S. saprophyticus* and is found in ~1–8% of urine isolates [62, 64] via the acquisition of a penicillin binding protein (**PBP**) with low β-lactam affinity encoded by the *mecA* gene [65, 66]. *mecA* is carried on the staphylococcal cassette chromosome (**SCC**) *mec* (**SCC*mec***) mobile genetic element (**MGE**) [50, 67, 68].

S. saprophyticus UTI virulence factors

As the most frequent Gram-positive causative agent of UTI, *S. saprophyticus* is also the best studied with respect to virulence determinants necessary to cause infection. Scanning electron micrographs of murine bladders infected with wild type *S. saprophyticus* show the organism adhering over the entire surface of the bladder but with apparent selective adherence to the tight junctions between individual epithelial cells [69]. Several adhesins have been linked to *S. saprophyticus* colonization of the urinary tract (Figure 1A). Conserved among all *S. saprophyticus* strains, Aas is a hemagglutinin that has autolytic and adhesive properties, binds to fibronectin and human ureters *in vitro, and* has been implicated in colonization of rat kidneys *in vivo* [70–74]. Also widely conserved, the surface-associated lipase, Ssp, is found in >90% of *S. saprophyticus* strains, forms fimbria-like surface appendages, and is important for acute UTI as well as persistent kidney infection in a murine

model [24, 74–76]. However, the mechanism by which Ssp contributes to *in vivo* infection is not well-understood.

Cell wall-attached surface proteins also mediate adherence in *S. saprophyticus* (Figure 1B). Sortase enzymes in Gram-positive bacteria covalently attach a subset of secreted proteins bearing sortase recognition motifs, or sorting signals, to the exterior cell wall [77]. Four sorting signal-containing proteins predicted to be cell wall-anchored via sortase enzymes have been described in *S. saprophyticus*: UafA, UafB, SdrI, and SssF. *S. saprophyticus* strains possess very few putative cell-wall associated sortase substrates compared to other staphylococci. The first sequenced *S. saprophyticus* strain ATCC 15305 encodes only one sortase substrate on its chromosome, UafA [78]. *S. saprophyticus* MS1146 encodes UafA, as well as UafB and SssF, on two different plasmids [79, 80]. SdrI was characterized on the unsequenced *S. saprophyticus* 7108 [81]. Uro-adherence factor A (UafA) is a hemagglutinin that mediates adhesion to bladder epithelial cells *in vitro* and is found in all *S. saprophyticus* strains examined to date [78]. Plasmid-encoded UafB, found in ~5% of strains examined, is a serine-rich glycoprotein that binds fibronectin, fibrinogen, and human bladder epithelial cells but does not promote bladder colonization in a murine UTI model [79]. Like the related serine-rich platelet binding proteins SraP in *S. aureus* and GspB in *Streptococcus gordonii*, which rely on an accessory SecA2 secretion system [82–84], UafB is encoded in a genetic locus that also contains gene for a putative accessory secretion apparatus [85]. In Gram-positive pathogens, these accessory Sec systems are associated with virulence [86]. SdrI is a cell wall-associated serine-aspartate-rich protein found in a minority of *S. saprophyticus* strains, that binds collagen, is associated with bacterial surface hydrophobicity, and plays a role in acute UTI and persistent kidney infections [24, 79, 81, 87]. SdrI shares sequence and structural homology with the adhesive Sdr proteins, including ClfA and ClfB, of *Staphylococcus aureus* and *Staphylococcus epidermidis* [81, 88, 89]. Plasmid-encoded SssF is highly conserved among *S. saprophyticus* strains, is involved in resistance to linoleic acid, but does not play a role in uropathogenesis in a murine model. Instead, SssF has been postulated to be important prior to urethral exposure, in biological niches such as the perineum or periurethral area where polyunsaturated fatty acids such as linoleic acid are particularly abundant [80].

In addition to cell-wall associated proteins, *S. saprophyticus* encodes urease that is important for efficient colonization of the bladder and kidneys, for inflammation in the bladder, and for dissemination to the spleen in a rat model of UTI [23]. The presence of urease-producing *S. saprophyticus* has been associated with the formation of urinary stones [90]. The ability of *S. saprophyticus* to tolerate high concentrations of D-serine that occur in the urine is conferred by D-serine deaminase, found in *S. saprophyticus* but in no other staphylococci [91] (Figure 1C). *S. saprophyticus* upregulates the virulence determinant Ssp in the presence of D-serine, and D-serine deaminase mutants are outcompeted by the isogenic wild type strain in the kidneys in a murine model of UTI [92]. *S. saprophyticus* express a capsule, whose encoding genetic locus in the sequenced strain is found on an SCC genetic element and whose genetic arrangement is similar to the *S. aureus cap5* (*cap8*) locus [78]. The capsule of *S. saprophyticus* mediates resistance to complement-mediated opsonophagocytic killing by human neutrophils [93] but also prevents binding to urothelial cells, perhaps via masking and preventing interactions of adhesin(s) with the cells [78]. Given the

contributions of capsule of other uropathogens, such as UPEC [94, 95], it will be of interest to determine at which stages of UTI this polysaccharide is acting.

Phenol soluble modulins (**PSM**) are staphylococcal pro-inflammatory cytolytic toxins characterized in *S. aureus* and *S. epidermidis*, are usually encoded within the core genomes, and are crucial in immune evasion as they are able to recruit, activate, and then lyse human neutrophils [96–99]. Many PSMs also have antibacterial activities alone or in cooperation with host antimicrobial peptides that can kill other microbes within a niche [100–102]. PSMs also mediate biofilm disassembly in their peptide form, whereas PSMs that assemble into amyloid-like extracellular fibrils under certain growth conditions promote biofilm stability [103–105]. One exceptional non-core genomic PSM has been found that is instead associated with the SCC*mec* MGE of *S. aureus*, termed PSM*mec*, genetically linking methicillin resistance and virulence [106]. PSM*mec* have been identified in other CoNS, including multiple human *S. saprophyticus* isolates [50, 106, 107]. A BLASTP search of the sequenced genome of methicillin-sensitive *S. saprophyticus* ATCC 15305 [78] for peptides homologous to PSMβ1 or PSMβ2 of *S. aureus* [96] revealed the presence of 3 putative PSM peptides with 55–80% identity to PSMβ1/2 that do not appear to be associated with MGEs (K.A. Kline, unpublished). Whether *S. saprophyticus* PSMs are expressed and functional in UTI remains to be experimentally determined.

Non-saprophyticus staphylococcal UTI

In contrast to *S. saprophyticus* which is a predominant cause of community acquired UTI, *S. aureus* UTI more often occurs in urinary-catheterized and pregnant individuals [108–110]. The majority of *S. aureus* UTI isolates are methicillin-resistant and *S. aureus* bacteriuria is associated with subsequent development of invasive infection [108]. Like *S. saprophyticus*, *S. aureus* also encodes an active urease enzyme. Two nickel ABC-transporters (Opp2 and Opp5a) have been identified as necessary for urease activity *in vitro*. These, along with a third ABC-transporter that imports nickel and cobalt when zinc is depleted, are both involved in UTI colonization and virulence in a mouse model [111, 112]. To our knowledge, no other *S. aureus* virulence factors have been examined during UTI.

Coagulase negative *S. epidermidis* is a member of the human skin microbiota and is an important opportunistic pathogen, especially in biofilm-associated infections associated with indwelling medical devices. Thus, the biofilm-forming properties of *S. epidermidis* are an area of active investigation and are summarized in a recent review [113]. CoNS, including *S. epidermidis*, are a lead cause of hospital-acquired infections where they are often methicillin resistant [114] and are associated with 2.5% of CAUTI [115]. In murine UTI studies in the absence of catheter, *S. epidermidis* is able to colonize the bladder at a similar frequency, but with significantly delayed kinetics, compared to *E. coli* or *S. saprophyticus* [69].

Host response to staphylococcal UTI

The immune response to *S. saprophyticus* UTI in humans is not markedly different from Gram-negative UTI and is characterized by symptoms such as lower urinary tract inflammation, pyuria, hematuria, and flank pain [49, 116]. In the mouse transurethral model of *S. saprophyticus* pathogenesis, inoculation of 10^7 bacteria into the murine bladder

resulted in the recovery of 100-fold more CFU from the kidney than the bladder as early as 6 hours post infection and for as long as 2 weeks post infection [24]. The kidney tropism of *S. saprophyticus* in a murine model may reflect the propensity of this organism to cause pyelonephritis in humans [49, 61]. The innate inflammatory immune response to *S. saprophyticus* UTI reflected the higher kidney titers observed, with ~100-fold greater induction of numerous pro-inflammatory cytokines and significantly more neutrophils infiltrating in the kidney compared to the bladder, all of which peaked at 48 hours post infection. Enhanced macrophage recruitment to the bladder was also observed during acute stages of *S. saprophyticus* UTI [24]. Recently, a role for the mitochondrial respiratory chain has been implicated in the innate immune response to *S. saprophyticus* infection. A mouse strain carrying a heterogeneous knock-out of a subunit protein of the mitochondrial complex I (**GRIM-19**) is prone to spontaneous urinary tract infection by *S. saprophyticus* [117]. In response to infection, macrophages from GRIM-19 mice produce lower amounts of pro-inflammatory cytokines and are defective for intracellular killing of *S. saprophyticus*. [117]. This report reflects this first mechanistic study of how the host handles *S. saprophyticus* infection.

Urinary Tract Infection caused by Enterococci

Epidemiology of enterococcal UTI

Enterococci are a genus of Gram-positive lactic acid bacteria that typically occur as diplococci or in short chains. These facultative anaerobes are γ-hemolytic and can tolerate a diversity of environmental conditions including temperature ranges from 10–45°C, pH ranges from 4.6–9.9, sodium chloride concentrations up to 6.5%, bile salts up to 40%, and desiccation despite the fact that do not form spores [118]. Enterococcus species *E. faecalis* and *E. faecium* are responsible for a minority of community-acquired UTI, but together cause 15 to 30% of catheter-associated UTIs and are the third leading cause of hospital-acquired UTIs [13, 115, 119]. Diabetic individuals are also at increased risk for UTI [120, 121], which serves as a nidus for the higher incidence of bacteremia in this population [122, 123]. While some studies indicate no increase in the frequency of enterococcal UTI in diabetic women compared to non-diabetic women [124, 125], others report that *Enterococcus spp.* are more often associated with UTI among diabetics and cause 13% of ASB in diabetics compared to 4.9% in non-diabetics [121, 126]. These differential findings in humans have been reflected in animal studies. In a chemically-induced (streptozocin) model of murine diabetes, diabetic mice were more susceptible to *E. faecalis* ascending UTI compared to non-diabetic mice [127], whereas pyelonephritis after intravenous injection of *E. faecalis* was similar in chemically-induced (alloxan) diabetic rats and non-diabetic rats [128]. Diabetes is also a risk factor for prostatitis, for which enterococci are responsible for up to 10% of cases [129, 130]. The incidence of UTIs due to *E. faecalis* has risen steadily over the years and *E. faecalis* UTI now outnumbers *E. faecium* UTI 5:1 [131]. Infection due to multiple-drug-resistant enterococcal strains presents a significant medical problem, with vancomycin resistance increasingly prevalent among *E. faecium* isolates [131]. *E. faecalis* readily adheres to and develops biofilms on abiotic surfaces such as urinary catheters. Many enterococcal virulence factors involved in UTI described to date are also biofilm

determinants. However, it is unclear whether these virulence factors function in a similar manner during biofilm formation and infection in the absence of abiotic devices.

E. faecalis UTI virulence factors

The *E. faecalis* surface protein Esp is a large ~200kD surface protein that is enriched among enterococcal bloodstream and endocarditis isolates compared to fecal isolates [132]. Esp is composed of multiple repetitive domains, sharing sequence similarity with Rib and C alpha virulence determinants in group B streptococci, which confer protective immunity and mediate immune evasion [132–135]. Esp promotes bladder colonization in a mouse model, as well as biofilm formation *in vitro* [136, 137].

Additional *E. faecalis* surface proteins that contribute to urovirulence in animal models include the collagen adhesin Ace and the enterococcal fibronectin binding protein EfbA [28, 138, 139]. Ace is regulated by the GrvRS two-component regulatory system and, accordingly, *gvrR* mutants are attenuated for biofilm formation *in vitro* and for virulence in a murine model of ascending UTI [140]. The ArgR family transcription factor AhrC is important for early biofilm formation *in* vitro, and *ahrC* mutans are significantly attenuated for biofilm growth on catheters as well as for bladder and kidney colonization during CAUTI [141]. Non-proteinacious *E. faecalis* factors monoglucosyl-diacylglycerol (MGlcDAG), diglucosyl-diacylglycerol (DGlcDAG), and D-alanylated lipotechoic acid (LTA) appear to limit UTI virulence because mutations in synthetic genes for each of these products increase colonization of urothelial cells *in vitro* and *in vivo*, suggesting that their expression interferes with the host-pathogen interaction in the urinary tract [142, 143].

Similar to other sortase-assembled pili in Gram-positive bacteria, biogenesis of the *E. faecalis* endocarditis and biofilm-associated pilus (**Ebp**) relies on a pilus-associated sortase (Sortase C in *E. faecalis,* SrtC) for pilus polymerization and the housekeeping Sortase A (**SrtA**) for cell wall anchoring [77, 144–147]. Temporal analysis of factors involved in biofilm formation *in vitro* showed that SrtA is important for the early attachment stage of biofilm formation, as is SrtC and the Ebp [144, 148]. Furthermore, SrtA and the Ebp are important for biofilm formation *in vivo* during CAUTI [30, 149]. However, since SrtA attaches multiple proteins to the cell wall, including aggregation substance [150] and Ebp, the contribution of SrtA may be attributable to multiple cell wall proteins in addition to the pilus. In contrast, *E. faecalis* SrtC and the Ebp, but not SrtA, are important for ascending UTI in the absence of catheter in an outbred mouse model [27, 151, 152] whereas Ebp is not required for ascending UTI in inbred mice [149]. An *E. faecium ebp* mutant is attenuated in the ascending UTI model [153]. Thus, it appears clear that *E. faecalis* Ebp is required for biofilm formation during infection, but its contribution to infection in the absence of foreign devices may be species dependent.

Ebp pili can bind extracellular matrix (**ECM**) proteins and human platelets *in vitro* [138, 154]. Recently, a predicted metal ion-dependent adhesion site (MIDAS) motif in the von Willebrand factor A (VWA) domain within the N-terminus of EbpA, the likely tip adhesin of the Ebp, was found to be essential for Ebp-dependent CAUTI, biofilm formation, and associated bladder colonization *in vivo* [155]. The VWA domain- and MIDAS motif-containing PilA of GBS, as well as the tip pilin RrgA of *Streptococcus pneumoniae*, bind

extracellular matrix proteins [156, 157]. The VWA domain of the PilA tip pilin of GBS is also important for bacterial adhesion to human alveolar and intestinal epithelial cells *in vitro*. The MIDAS motif of *E. faecalis* EbpA mediates binding to host fibrinogen, which is released in the bladder in response to catheter implantation and subsequently coats the catheter surface, providing a possible explanation why *E. faecalis* is frequently associated with CAUTI [158].

Immune responses to enterococcal UTI

In a murine model, *E. faecalis* can colonize and persist in the kidneys, but is rapidly cleared from the bladder in the absence of apparent inflammation [26], suggesting lack of adherence by *E. faecalis* in the bladder. Nevertheless, urothelial cells containing intracellular *E. faecalis* have been observed in the urine of patients displaying lower urinary tract symptoms (LUTS) such as pain and issues associated with urine storage and voiding, and the same *E. faecalis* strains can invade human urothelial cells *in vitro* [159]. Kidney tropism is a common theme among Gram positive bacteria in the murine infection model [24, 25]. Mild inflammation observed in the infected kidneys at 2 days post infection, consisted primarily of monocytic cells and neutrophils, and was insufficient to clear the kidney infection. Aggregation substance promotes *E. faecalis* survival within human neutrophils [160]. *E. faecalis* can also survive longer within macrophages compared to non-pathogenic organisms such as *E. coli* DH5α and *Lactococcus lactis* [161] and it has been proposed that glycosaminoglycans on the macrophage surface are receptors for *E. faecalis* [162]. A number of enterococcal factors have been implicated in survival within macrophages, including extracellular polysaccharide, Ace, and oxidative stress responses [139, 162–166]. In addition, methionine sulfoxide reductases A and B, predicted to reverse protein oxidation, not only promote survival within activated murine peritoneal macrophages but also during ascending UTI [167]. Together these studies present a picture in which macrophages are a key responder to *E. faecalis* UTI and where survival within macrophages may be important for the ability of enterococci to persist within this niche.

The host signaling cascades leading to *E.* faecalis-mediated immune infiltration into the urinary tract is not yet well-delineated. In mice, toll-like receptor 2 (TLR2), which recognizes lipopeptides to initiate innate immune responses to Gram-positive bacteria, is not involved in the host response to *E. faecalis* either in TLR2-transiently transfected 293 cells *in vitro* or in the murine urinary tract [26, 168]. However, *E. faecium*-induced signaling in murine peritoneal macrophages requires both TLR2 and the intracellular adaptor protein MyD88 involved in TLR-mediated signaling, and these are thought to be important for signaling neutrophil recruitment during experimental peritonitis [169]. After *E. faecalis* uptake into macrophages, pro-inflammatory signaling cascades can also be induced via interaction between the intracellular macrophage nucleotide-binding oligimerization domain 2 (**Nod2**) protein and *E. faecalis* peptidoglycan fragments [85].

In contrast to ascending UTI, CAUTI is rarely symptomatic [15]. Moreover, urinary catherization alone, in the absence of bacterial infection, can give rise to dysuria, urinary urgency, urothelial damage, and bladder edema [15, 170–172]. Despite being commonly asymptomatic, CAUTI is accompanied by pyuria. However, Gram-negative associated

CAUTI is more strongly associated with pyuria than those caused by Gram-positive organisms. CAUTI associated with Gram-positive organisms such as enterococci or CoNS are less inflammatory, as measured by leukocytes in the urine, compared to CAUTI associated with Gram-negative bacilli [173]. These features of Enterococcal CAUTI are also reflected in the murine CAUTI model where implantation of a foreign body (catheter) alone causes major histopathology including edema, urothelium damage, and proinflammatory cytokine expression. *E. faecalis* can overcome the robust catheter-mediated inflammatory response and replicate to high numbers as a biofilm on the catheter, as well as in the bladder and kidney [31]. Despite high *E. faecalis* CFU during CAUTI, markers of inflammation are not greatly enhanced in mice receiving the catheter implant together with *E. faecalis* compared to animals receiving catheter alone [30]. Infection of catheterized mice with *E. faecalis* results in a modest augmentation of inflammation in the bladder, characterized by a more than 2-fold increase of interleukin 1β (IL-1β) and macrophage inflammatory protein 1α (Mip-1α), despite bacterial loads in the bladder exceeding 10^6 CFU [30]. These, differential inflammatory responses to enterococcal CAUTI versus ascending UTI may be due, in part, to differing responses of macrophage to *E. faecalis* in a biofilm state compared to planktonic cells *in vitro* [174]. In addition, there is a significant decrease in the number of infiltrating activated macrophages during *E. faecalis* CAUTI compared to catheterization in the absence of bacteria suggesting an immunosuppressive capacity of *E. faecalis* [31]. The gene encoding a Toll/interleukin-1 receptor (TIR) domain containing protein (*tcpF*) is enriched among *E. faecalis* UTI isolates and can downregulate the host inflammatory response, presumably by interfering via molecular mimicry with the TIR-TIR interactions between TLRs required for TLR dimerization and subsequent signaling [175, 176]. Consistent with this finding, several studies of *E. faecalis* strains isolated from the gastrointestinal tract of healthy human infant guts have shown that a subset of strains were capable of suppressing inflammatory cytokine expression in human intestinal epithelial cells *in vitro*, as well as suppressing cytokine responses in a Dextran Sulphate Sodium salt (DSS) model of inflammatory intestinal colitis *in vivo*, although the presence of *tcpF* was not examined in these strains [177–179]. Together these studies indicate that high titer *E. faecalis* CAUTI is not highly inflammatory and can be immunosuppressive.

Urinary Tract Infection caused by Group B *Streptococcus*

Epidemiology of GBS UTI

Streptococcus agalactiae, otherwise known as group B *Streptococcus* (GBS) is a Gram-positive β-hemolytic chain-forming coccus that is a common asymptomatic inhabitant of the lower gastrointestinal and female reproductive tracts. GBS is estimated to cause approximately 1–2% of all monomicrobial UTIs [180]. Other studies of elderly populations with UTI show an involvement of GBS in as many as 39% of nursing home residents over 70 years of age [181]. Asymptomatic bacteriuria (ASB) and UTI caused by GBS are common not only among the elderly, but also in pregnant, diabetic, and immunocompromised individuals, as well as those with pre-existing urologic abnormalities, groups with higher risk of ascending pyelonephritis that can progress to bacteremia and/or urosepsis [10, 182–184]. While GBS may represent only a small fraction of total UTIs, the

burden of GBS UTI is a major public health concern [10], with approximately 160,000 cases annually in the U.S.

Among nonpregnant adults, the incidence of systemic GBS infections is estimated at approximately 4.4 cases per 100,000 individuals; 14% of these are cases of urosepsis [185]. Common underlying conditions of individuals with GBS urosepsis include diabetes mellitus, malignancy, chronic kidney disease, recurrent urinary tract infections, obstructive neuropathy, and neurogenic bladder [185, 186]. In about 30% of cases, systemic infections caused by GBS in nonpregnant adults do not have an apparent focal origin such as cellulitis, pneumonia, or UTI. In contrast to the proportion of UTIs associated with GBS in young nonpregnant populations, estimated at 1–2% [180], it has been estimated that up to 7% of pregnant women have significant titers of GBS in urine [187, 188].

GBS bacteriuria in pregnancy

GBS is the leading cause of sepsis and meningitis in newborns and can be acquired by the newborn *in utero* or during passage through the colonized birth canal. In addition to colonizing the reproductive tract, GBS is often found to colonize the urinary tracts of pregnant women. Although GBS colonization of the urinary tract in pregnancy is often asymptomatic, GBS bacteriuria is an independent risk factor for maternal pyelonephritis and chorioamnionitis as well as neonatal GBS sepsis [187, 189–192]. The Centers for Disease Control recommend universal screening of GBS vaginal-rectal colonization at 35–37 weeks of gestation and antibiotic prophylaxis for culture-positive women during labor and delivery.

Screening and treatment for ASB is also recommended in pregnancy, since women with ASB are at higher risk of preterm delivery, have a 20–30-fold increased risk of pyelonephritis, and antibiotic treatment of ASB has been demonstrated to reduce these risks significantly [193–198]. The 2002 recommendations from the CDC stated that clinical microbiology labs should report *any* concentration of GBS detected in urine. The 2010 revised guidelines notes that this practice represents an

> "increased workload for clinical microbiology laboratories, which do not generally report bacterial growth in urine of other pathogens at concentrations $< 10^4$ cfu/ml and rarely know whether urine samples are from pregnant women; as a result, some laboratories search for any GBS colonies in urine cultures from all women of reproductive age [199]."

Surprisingly, there is little published clinical data investigating whether low GBS titers ($< 10^4$ CFU/ml) in urine is associated with adverse maternal or neonatal outcomes. One study found that babies of women with low titer GBS bacteriuria were at higher risk of GBS disease compared to women without detectable GBS in urine [200]. Other studies have not been performed to confirm or refute this finding, but it has been argued that this study *may* have been biased because only a subset of the women underwent urine culture [199]. Clearly, additional studies are required to estimate the threat of low-level GBS in urine for neonatal GBS disease and determine the best course of action for screening and prophylactic measures. One study estimated that 4% of women who test negative for GBS rectal or vaginal colonization test positive for GBS in urine culture [201], suggesting that the urinary tract could be a distinct and independent niche for the bacterium that could be missed in

routine third trimester screening procedures. Currently, the CDC recommends that women with GBS bacteriuria at any time in pregnancy should be given prophylactic antibiotics at the time of labor and delivery.

Invasive GBS disease in the elderly

UTI caused by GBS is approximately 10-times more frequent than GBS neonatal infections and is common among the elderly. Case fatality rates for invasive GBS infection are also higher among the elderly (~15%) compared to young infants with invasive infection (4–6%) [10]. However, in contrast to screening and prophylactic measures during pregnancy and post-partum periods, similar screening and prevention strategies are lacking in settings where elderly patients are at risk of invasive GBS disease. Attempts to develop an effective GBS vaccine have been successful in early trials in animals and humans [202–209], raising the possibility of future reductions in the incidence and severity of GBS disease in at-risk groups.

GBS virulence factors and induction of host immune responses

GBS can cause infections at a variety of body sites (skin, soft tissue, lung, peritoneum, urinary tract, etc.) and utilizes a wide range of virulence factors to injure and invade host tissues, resulting in disseminated infection (e.g. bacteremia, osteomyelitis, meningitis) [210]. Although much is known about the molecular epidemiology of GBS, the organism has not been studied extensively in animal models of UTI and thus, relatively little is known about which virulence factors may play important roles in this context.

Epidemiological analyses have shown that a variety of GBS serotypes are associated with UTI. However, serotype III GBS was found to cause a disproportionate number of acute symptomatic disease compared to other serotypes, which were more likely to be associated with asymptomatic bacteriuria [183]. These clinical studies have formed the basis for experimental studies of GBS urinary tract infections using serotype III GBS strains in mouse models of transurethral infection [25, 211, 212]. In the murine model, the presence of GBS robustly induces IL-1α [212], macrophage inflammatory protein-1α (MIP-1α), MIP-1β, IL-9, and IL-10 [25]. However, experimental studies have observed a striking lack of overall histological inflammation in the bladder during GBS cystitis [25] and marked differences in transcriptional responses compared to *E. coli* UTI [211]. A number of GBS virulence factors have been characterized in other models, but only a few studies have examined GBS virulence factors by inoculating wild type and mutant strains of bacteria into the mouse urinary tract [25, 39, 213]. These studies have demonstrated that sialic acid residues of the GBS capsular polysaccharide are necessary for optimal establishment of GBS in the urinary tract [25], whereas the β-hemolysin/cytolysin did not appear to have a significant effect on bacterial survival following transurethral infection [25, 213]. Additional studies are needed to identify GBS virulence factors of importance in the urinary tract and to understand the cellular, molecular, and biochemical details of host-microbe interactions in populations at-risk for GBS disease.

In the remainder of this chapter, we will 1) discuss several emerging, rare and/or underreported Gram-positive pathogens of the urinary tract, 2) introduce several recent

studies that demonstrate some unexpected inhabitants of the urinary tract using culture-independent approaches for bacterial detection in urine, and 3) examine evidence that host urogenital colonization states may influence the risk of UTI.

Vaginal Microbiota, Bacterial Vaginosis (BV) and UTI

Mounting clinical evidence argues that the composition of a woman's vaginal microbiota influences her risk of UTI. Women with a dominant population of vaginal lactobacilli are at a lower risk of UTI compared to women with more diverse microbiota, consisting of Gram-negative anaerobes, *Actinobacteria*, and other *Firmicutes* [214–216]. This vaginal condition is referred to by some as bacterial vaginosis (BV) and is often labeled as an 'imbalance' or dysbiosis of the vaginal microbiota because it has been associated with a wide variety of adverse health outcomes for women and their babies [217, 218]. Clinical features of BV (i.e. Amsel criteria) include vaginal pH >4.5, 'thin' grayish homogenous vaginal fluid, fishy odor upon KOH treatment of vaginal fluid and the presence of epithelial cells studded with bacteria in wet mount (i.e. 'clue-cells') [219–221]. Another method for BV diagnosis that has been used more extensively in recent years is the Nugent method [220], which is based on a morphotype scoring system using Gram-stained slides where a higher score indicates BV. Only recently have studies in experimental models demonstrated that a single bacterium, *Gardnerella vaginalis*, one of the common bacteria to overgrow in BV, is sufficient to yield clinical features and biochemical phenotypes of BV in a murine vaginal infection model [222, 223]. Studies examining the association between BV and UTI have found that women with BV have anywhere from a 2.2- to 13.7-fold increased risk of UTI depending on the population studied [214–216]. Further supporting these findings, clinical studies now provide compelling preliminary data that vaginal probiotic administration may be beneficial for women who are prone to recurrent UTI [224]. These studies are consistent with results of anaerobic culture studies presented above, showing that women with recurrent UTI were more likely to contain higher titers and a more diverse repertoire of mixed anaerobes in their urine than healthy (sexually active or inactive) women [225]. Taken together, these results suggest a possible linkage between dysbiosis of the vaginal microbiota and dysbiosis of the bladder microbiota.

Unfortunately, our understanding of *why* women with bacterial vaginosis are more prone to urinary tract infection is limited. It is thought that lactic acid, hydrogen peroxide, and other 'defensive' molecules produced by lactobacilli may create a hostile environment for potential pathogens, including uropathogens, in the vagina [226–228]. However, experimental studies are needed to more fully understand the causal relationships linking specific vaginal bacteria with UTI susceptibility in experimental models. While it remains a distinct possibility that lactobacilli act through specific mechanisms to discourage UTI [229], another possibility (though not a mutually exclusive one) is that one or more taxa of BV bacteria act through unknown mechanisms to enhance UTI susceptibility. As described below and illustrated in Table 1, **and** Figure 2 (with emphasis on Gram-positive bacteria) many of the bacterial genera that have been described in the BV-associated vaginal environment have also been observed in both the female and male urinary tracts using culture-independent approaches. These findings raise the possibility that fastidious

organisms associated with BV could play a more direct role in the etiology of host-uropathogen interactions within the urinary tract.

Rare, Emerging, and Under-reported Gram-Positive and Polymicrobial Etiologies of UTI

In the next section of this chapter, we present several specific examples of Gram-positive bacteria that are rare, emerging, or underreported, including species of *Aerococcus, Corynebacterium, Actinobaculum*, and the potential uropathogen *Gardnerella vaginalis*. These organisms may be missed as causes of UTI due to 1) misclassification due to lack of distinguishing phenotypic criteria, 2) dismissal of significant growth as 'microbiota contamination', or 3) lack of detection by standard approaches.

Aerococcus as a cause of UTI

Aerococcus is a genus of microaerophilic, facultatively anaerobic, α-hemolytic, Gram-positive cocci that are catalase- and oxidase-negative and leucine aminopeptidase positive. One unique characteristic of aerococci is that they divide on 2 planes at right angles, resulting in tetrads and irregular clusters. Species of *Aerococcus* are commonly isolated from air, dust, and vegetation, and are also common isolates from the human vagina and urinary tract. Several species of *Aerococcus* can cause urinary tract infections and urosepsis, including *A. urinae, A. viridans*, and *A. sanguinicola* [230–236]. One study reported that 0.8% of all urine specimens cultured during a 4-month period in a Denmark hospital yielded growth of "*Aerococcus*-like" organisms [237]. Patients with UTI caused by *Aerococcus* are most often elderly and many have urological abnormalities or other risk factors for UTI [230, 233, 237]. Many of the cases described are invasive systemic infections in which *Aerococcus* is isolated from blood along with significant *Aerococcus* titers in urine.

Aerococci have a number of biochemical and physiological similarities with lactococci, pediococci, enterococci, and streptococci [238]. In particular, phenotypic similarities between *Aerococcus* and viridans group streptococci have made it difficult for clinical labs to distinguish between them using routine phenotypic tests. For example, one study examined the ability of three commonly used bacterial identification systems (API 20 STREP, ID 32 STREP, and VITEK 2 ID-GPC card, bioMérieux) to correctly identify 30 urinary tract isolates representing different species of *Aerococcus*. This study revealed that *Aerococcus* isolates (with the exception of *A. viridans*) were commonly misidentified or identified with low discrimination [239]. Molecular tools such as amplification and sequencing of 16S rRNA, which are not commonly employed in clinical microbiology labs, are needed for accurate identification of *Aerococcus* species. The result is that aerococci are often misclassified and/or discarded as likely contaminants.

Prompt and accurate identification of *Aerococcus* is necessary to avoid life-threatening systemic infection by this potential pathogen in susceptible individuals that present with uncomplicated UTI. Most isolates of *Aerococcus urinae* have been characterized as resistant to sulfonamides [240, 241]. Case reports of *A. urinae* UTI demonstrate that when patients are treated with antibiotics effective against the organism, bacteriuria cleared and the patient

recovered fully; however, in other situations when *A. urinae* was not recognized or effective antibiotic treatment was not promptly provided, patients often progressed from simple UTI to invasive systemic infections [234, 235, 240, 242, 243].

Corynebacterium urealyticum

Corynebacteria are Gram-positive, non-motile, non-spore forming, facultatively anaerobic *Actinobacteria* that are common components of the skin microbiota and increasingly recognized as opportunistic pathogens [244, 245]. *Corynebacterium urealyticum* has been associated with asymptomatic bacteriuria, and rarely, with acute and chronic infections of the urinary tract. *C. urealyticum* (previously known as *Corynebacterium* group D2) is the most common cause of alkaline encrusting cystitis and pyelitis, a chronic inflammatory condition of the urinary tract in which the bacterium causes painful localized ulcerations with deposits of ammonium magnesium phosphate (a.k.a. struvite) that can be visualized on plain radiography [246_248]. Alkaline urine and the presence of struvite crystals in urine sediments are characteristic features of *C. urealyticum* encrusting cystitis and occur due to bacterial expression of urease activity, which catalyzes the conversion of urea to ammonia and carbon dioxide. The presence of a previous urological procedure and/or mucosal lesion appears to be necessary for urea-splitting bacteria such as *C. urealyticum* to cause encrusting UTI. Immune compromised patients with underlying urologic disease, those that have undergone long-term hospitalization and/or previous treatment with broad-spectrum antibiotics are also at greater risk for developing alkaline encrusted UTI. While this type of UTI is considered rare, one prospective study showed that 9.8% of renal transplant recipients (n=163) had *C. urealyticum* in urine, with nearly half of positive patients experiencing no symptoms until at least 1 month after first detection of the organism [249].

C. urealyticum infection can often be missed in routine urine culture because the organism is slow growing and requires enriched media. Moreover, similar to other *Actinobacteria, C. urealyticum* requires at least 48 hours of growth on blood agar plates to produce pinpoint colonies, which may be dismissed as "contaminating microbiota," since it is generally thought that species of *Corynebacteria* other than *C. diptheriae* are nonpathogenic. The presence of alkaline urine or struvite crystals in urine sediment should prompt cultures of longer duration. A number of cases of *C. urealyticum* UTI also report that Gram-staining of urine specimens reveals Gram-positive bacilli and polymorphonuclear cells, despite routine urine cultures that come back negative. Molecular detection techniques can also be employed when tests are negative, but there is strong suspicion of encrusting cystitis or pyelitis with *C. urealyticum*. Encrusting cystitis caused by *C. urealyticum* is treated by endoscopic removal of encrustations, acidification of the urinary tract, and treatment with appropriate antibiotics. A number of reports document resistance of *C. urealyticum* isolates to ampicillin, cephalothin, gentamicin, imipenem, tetracycline, ciprofloxacin, ofloxacin and sometimes to tetracycline, erythromycin, and rifampicin [250, 251]; however, most isolates were susceptible to synercid and linezolid [250].

Actinobaculum schaalii

The genus *Actinobaculum* and the species *Actinobaculum schaalii* were first described in 1997 following the isolation of organisms described as *Actinomyces*-like or

Corynebacterium-, like from human blood and urine that displayed >6% sequence divergence from the nearest relative, *Actinobaculum suis* [252], a swine uropathogen also previously known as *Actinomyces suis, Corynebacterium suis* or *Eubacterium suis* [253–255]. Species of *Actinobaculum* are members of the family *Actinomycetacea,* which also includes the genera *Actinomyces, Aracanobacterium*, and *Mobiluncus* [256]. These bacteria are facultative or obligate anaerobes and their detection in clinical specimens often requires a full 48 hours of growth on blood agar plates in the presence of a 5% carbon dioxide atmosphere. Phylogenetically, these bacteria belong to the high G content Gram-positives, but phenotypically, they can appear 'Gram-variable' since their cell walls are relatively thin compared to other Gram-positive bacteria and thus more easily decolorized during the Gram-staining procedure. Currently, the genus *Actinobaculum* contains four species: *A. suis, A. schaalii, A. massiliense, and A. urinale.* To date, *A. urinale* and *A. massiliense* have only been described in a few cases of human pathology, including urinary tract infections. In contrast, *A. suis* is a well-documented uropathogen of female pigs [254, 257–259] that appears to colonize the prepuce in a large proportion of wild and domesticated adult males [255, 260]. Here we focus on *A. schaalii,* which appears to be an emerging human uropathogen, particularly among the elderly and those with underlying urologic abnormalities [261].

A. schaalii is a small rod-shaped (straight or slightly curved and sometimes branching), nonmotile, nonsporulating, weakly β-hemolytic, facultative anaerobe requiring CO_2 for growth and testing negative for catalase, oxidase, urease, and nitrite production. *A. schaalii* is resistant to trimethoprim and ciprofloxacin, antibiotics used as first-line treatments for urinary tract infection. Although the natural history surrounding this species is still unclear, culture-dependent and –independent studies published to date suggest that this bacterium may be a commensal inhabitant of the human urinary tract. To date there have been approximately 130 reported cases of human infection with *A. schaalii,* mostly as small retrospective studies and case reports [252, 261–279]. Approximately 85% of *A. schaalii* infections reported so far occur in the urinary tract (i.e. single organism cystitis, pyelonephritis, or urosepsis) or have disseminated (e.g. bacteremia, endocarditis) from an unknown primary source. These infections most often occur in elderly individuals and those with underlying urologic conditions such as chronic renal failure or urologic obstruction [261]. In clinical cases of *A. schaalii* UTI, Gram-positive bacteria are often evident by direct microscopy, and yet, typical aerobic urine culture often produces a negative result [266, 268]. Cases of apparent urosepsis where *A. schallii* was isolated using anaerobic blood culture techniques were documented only after culture conditions were altered, eventually leading to the identification of *A. schaalii* in urine.

A. schaalii has been considered an extremely rare uropathogen. However, recent literature suggests that *A. schaalii* may be underreported due to inadequate culture and identification techniques. Due to fastidious growth conditions required for *A. schaalii* culture, and the similar appearance of the colonies to resident microbiota of the skin and genitourinary tract, it is likely this organism has been overlooked or dismissed as "contamination" when it may be clinically significant in certain settings [252]. Traditional phenotypic tests are still inadequate for identification of *A. schaalii.* Instead, studies have successfully used the API Coryne and Rapid ID 32A strip test systems (bioMérieux) together with molecular

methodologies (sequencing of 16S ribosomal DNA and/or species-specific primers for quantitative PCR) for identification and/or enumeration of *A. schaalii.*

A recent study examined 252 randomly selected urines from individuals in 3 hospitals in Viborg County, Denmark using a validated quantitative polymerase chain reaction that specifically analyzed the presence and titers of *A. schaalii*. The authors of this study found that 22% of persons >60 years of age harbored at least 10^4 CFU/ml of urine [268]. Additional studies are needed to estimate the prevalence of *A. schaalii* in the urinary tract in other populations and to define the possible clinical significance of this organism.

Gardnerella vaginalis

Gardnerella vaginalis belongs to the class *Actinobacteria* (also frequently called 'high GC Gram-positives') and the order *Bifidobacteriales. G. vaginalis* is best known for its connection to bacterial vaginosis (BV), a condition marked by *G. vaginalis* overgrowth in the vagina. BV has been linked to higher risks of a wide variety of adverse women's health outcomes, including UTI [214–216, 280, 281]. In addition to the ever more apparent role of *G. vaginalis* in BV, a growing body of evidence implicates the organism as a potential uropathogen. *G. vaginalis* (see Figure 3 for images of this organism) is a fastidious bacterium that cannot be recovered under the same conditions as typical uropathogens. It requires incubation in the presence of 5% CO_2 or better yet, under anaerobic conditions. Although it can be recovered on sheep blood agar under these conditions, the organism cannot survive in acidic urine or in urine stored at room temperature or at 37°C (rather than refrigerated) prior to recovery. *G. vaginalis* prevalence is significantly underestimated if plates are incubated for only 24 hours, but instead requires extended incubation (48–72 hrs) [282].

In two large studies encompassing culture analysis of a total of ~33,000 urine specimens, *G. vaginalis* was reported in 0.6–2.3% of all samples at titers >10^4 CFU/ml of urine. *G. vaginalis* was often identified in pure culture and in a context of patient reported symptoms and/or pyuria [283, 284]. One of these studies (reporting *G. vaginalis* in 0.6% of urines) only examined plates at the 24 hour time point [284], suggesting that 0.6% may be an underestimation. The larger of these two studies examined hospital inpatients and reported that among the patients with *G. vaginalis* bacteriuria, 58% had evident pyuria and 10% had pyelonephritis [285]. These patients were also more likely to have a history of recurrent UTI. Other studies provide further support that *G. vaginalis* presence in urine is not simply the result of periurethral or vaginal contamination and further suggest a possible role of *G. vaginalis* in renal disease. Several studies in the 1960's-80's identified *G. vaginalis* identified in urine isolated by suprapubic aspiration, thus bypassing possible urogenital contaminants [286–290]. These studies suggest that pregnancy renders women more likely to harbor *G. vaginalis* in their bladders and that recovery of the organism was especially common in women with underlying renal disease. Another study described *Gardnerella* as common isolate from aspirates of individuals with reflux scarring and so-called "sterile pyelonephritis" [291]. In addition to these culture-based studies, more recent studies using culture independent approaches have echoed these early studies. In fact, one study in

particular concludes that *G. vaginalis* is found at high levels in urines collected by suprapubic (needle) aspiration from many older adult women [292].

In addition to its apparent ability to infect the bladder and kidneys, *G. vaginalis* has also been described in bloodstream infections. One study reported thirty cases of bacteremia caused by *G. vaginalis* among obstetric patients over a 4-year period and suggests that bacteremia caused by this organism may be significantly underreported [293]. Although cases of *G. vaginalis* bacteremia are enriched in the gynecologic setting, occurring after birth [294_296] or following procedures such as endometrial ablation [297] or vaginal myomectomy [298], *G. vaginalis* bloodstream infection is not limited to women [299_304]. Indeed, the evidence suggests that female to male transmission of *G. vaginalis* can occur during sexual encounters and that regular condom use reduces the likelihood that *G. vaginalis* can be isolated from male urine [305, 306]. In one example of bloodstream infection in a male, a previously healthy individual with flank pain was found to have urolithiasis (kidney stones) with fever, elevated peripheral neutrophils, and elevated creatinine (indicating damage to the kidneys) [301]. Blood cultures revealed the man had urosepsis caused by a coccobacilli that produced pinpoint grey colonies on chocolate agar. In this case the hospital microbiology lab was not equipped to identify the organism. Instead, a national microbiology laboratory performed an extensive workup, identifying the organism as *G. vaginalis*. Another example involved an uncircumcised man with a previous history of diabetes mellitus and an ongoing sexual partner with recurrent BV. The man was shown to have bloodstream *G. vaginalis*, infective endocarditis, along with apparent septic emboli on one kidney and in the brain [300]. In this case, routine culture of the urine was also unable to identify *G. vaginalis* as the culprit.

G. vaginalis has also been described in polymicrobial infections in individuals with underlying urologic abnormalities or following urologic procedures. One case series describes *G. vaginalis* as commonly co-occurring with members of the genus *Bacteroides* in abscesses following urological procedures [285]. Sturm concluded this case series by recommending that microbiology labs should perform Gram-staining of urine specimens and should follow up results of pyuria in the context of visible coccobacilli by performing culture under conditions capable of *G. vaginalis* recovery. Given the apparently high risk of *G. vaginalis* infection following urological instrumentation, Sturm suggests that routine culture for this organism may be warranted during preoperative testing.

The role of *G. vaginalis* as a genitourinary pathogen is still controversial. Recent work in an experimental model of vaginal infection with *G. vaginalis* demonstrated that strain JCP8151B, isolated from a woman with BV, was sufficient to induce features of BV in mice [110]. This is the first animal model in which any BV bacterium has been shown to induce the formation of "clue" cells – exfoliated vaginal epithelial cells coated in bacteria – one of the hallmark microscopic features of BV. Interestingly, multiple studies of urinary tract infections involving *G. vaginalis* report the presence of clue-like cells in urine, both from women [291] and from men [307_309]. Interestingly, a wide variety of genera from the phylum *Actinobacteria* have been implicated in UTI or identified as part of the urinary microbiome, including not only *Gardnerella,* but also *Actinobaculum, Corynebacterium, Actinomyces, Atopobium, Alloscardovia,* and *Bifidobacterium* (see Figure 3 for images of

these bacteria). Few of these genera are accepted as true uropathogens. Thus, experimental studies of infection in animal models are needed to help settle the debate about their potential roles as uropathogens, either in the classical sense or by predisposing the urinary tract to infection or other urologic diseases.

Urine "contamination" with normal microbiota or polymicrobial UTI?

The potential biological and clinical significance of polymicrobial growth in urine depends on many factors. On one hand, the finding of multiple colony types after urine culture is a valid and justifiable concern to suspect contamination of the urine specimen with periurethral and/or vaginal microbiota. For this reason, most clinical microbiology labs will not evaluate plates with polymicrobial growth, but rather dismiss them as "contamination" and request another specimen. For instance, in suspected cases of uncomplicated cystitis, *E. faecalis* and GBS are often assumed to represent contamination of the urine specimen originating from the periurethral area during collection [1]. In fact, one recent study evaluated titers of these Gram-positives in midstream urine and compared to titers obtained when the same women were subjected to catheterization for urine collection. This study concludes that "enterococci (in 10% of cultures) and group B streptococci (in 12% of cultures) were not predictive of bladder bacteriuria at any colony count (Spearman's r=0.322 for enterococci and 0.272 for group B streptococci)." However, only a few patients in this study had *Enterococcus* or GBS at levels considered significant for a UTI diagnosis (>100,000cfu/ml). Further studies are needed to define 1) the relationship between Gram-positive bacteria in midstream and catheter-collected urine when patients meet the threshold for UTI (10^5 CFU/ ml), 2) the likelihood of cystitis when these organisms are detected in pure culture versus in the context of multiple other colony types, and 3) whether these relationships are similar across all patient groups. We caution against dismissing Gram-positive pathogens as unimportant. For example, while *S. saprophyticus* is now established as the predominant Gram-positive uropathogen, it was originally considered to be a urinary contaminant [310, 311].

There are multiple cited cases of polymicrobial bloodstream infection with identical organisms present in both urine and blood cultures [for examples see [312_314]]. In these cases, polymicrobial bloodstream infection supports the interpretation of polymicrobial UTI, especially when multiple of the organisms identified in the blood are also identified in urine. Moreover, since cases of disseminated polymicrobial infection occur most often in compromised persons with underlying risk factors for UTI, the organisms involved are more likely to be members of the 'normal' microbiota that may be overlooked in urine specimens as nonpathogens. In another related case, a woman received a suprapubic catheter following urogynecological surgery and later developed an abscess near the location of bladder catheter insertion. Although hospital microbiology lab returned the result of this urine culture as "contaminated," identification of bacteria from the anaerobically cultured surgically drained pus demonstrated a polymicrobial infection with *E. coli, G. vaginalis,* and *Peptostreptococcus productus* [283].

It has been estimated that up to 20% of women presenting with classic symptoms of UTI have culture-negative urine [315]. In the cited example "culture-negative" included samples

without a single dominant species (i.e. polymicrobial growth), samples with "secondary pathogens" at titers $<10^4$/ml urine, or "doubtful pathogens" at titers $<10^5$ CFU/ml urine. Unfortunately, the term sterile is often applied incorrectly to these "culture-negative" contexts. For example, Domann et al. found that 9.2% of urine specimens collected from renal transplant recipients did not produce significant growth under aerobic conditions, but had evidence of intact bacterial rods and/or cocci that were identified as fastidious anaerobes using culture-independent molecular approaches [316]. Alternatively, what might appear to be a monomicrobial infection when observed by aerobic culture may actually be a polymicrobial infection when characterized by culture-independent techniques. For example, one recent study detailed a case study where a woman had what appeared to be a typical culture-positive UTI with $>10^5$ CFU/ml of monomicrobial *E. coli* according to aerobic clinical lab results. The culture-independent approach performed in parallel revealed that urine specimens collected by catheterization and suprapubic aspiration from this patient contained *Actinobaculum* and *Aerococcus* at levels that far exceeded *E. coli* [317]. Other studies that have examined the incidence and significance of *Actinobaculum* and *Aerococcus* in urine specimens have shown that up to 90% and 69% of the time, respectively, significant titers of these organisms were found alongside significant titers of typical uropathogens such as *E. coli* [230, 268].

Another recent study performed anaerobic culture for fastidious anaerobes in urine of women who suffer from recurrent chronic episodes of cystitis and compared these results to a similarly aged groups of young women without UTI [225]. The study reports that women with recurrent cystitis were more likely to contain higher titers and a more diverse repertoire of mixed anaerobes in their urine than healthy women without UTI. In fact, this study had two healthy control groups: one with women who reported regular sexual activity and another with women who reported no history of sexual intercourse. Results of the study strongly suggest that sexual activity does not provide a simple explanation for why women with recurrent chronic cystitis have fastidious bacteria in their urine. Taken together, these findings suggest that fastidious organisms and species assumed to represent contamination of urine specimens in urine cultures are routinely overlooked. We argue that additional studies specifically evaluating contested potential uropathogens in specific patient settings such as those who suffer from recurrent UTI, those at risk for complicated UTI, and those with other urologic problems of uncertain etiology (e.g. interstitial cystitis, urinary urgency incontinence, preeclampsia etc) are still needed.

"Uncultivated" Bacterial Inhabitants of the Urinary Tract

Clinical microbiology labs employ various combinations of culture-dependent approaches in the analysis of urine specimens. These approaches include the use of selective and nonselective agar media for enumeration and identification of bacteria under *aerobic* conditions. Incubation of agar plates is typically performed at 35–37°C for 18–24 hours. Some labs use additional approaches to cast a slightly wider net to avoid false-negative results, often depending on clinical findings or other factors, extending aerobic incubation to 2–3 days, incubating plates in the presence of 5% CO_2, or performing Giemsa- or Gram-staining of urine specimens. In the last few years, culture-independent approaches have evaluated whether the urinary tract contains microbes that are not cultivated under typical

aerobic conditions used for urine culture. Table 1 summarizes the results of the urinary tract microbiome studies that have been performed to date with an emphasis on Gram-positive organisms observed using culture-independent approaches (see right side of Table, 'culture-independent' detection).

In one study, Domann et al. compared the use of traditional urine culture to the use of a culture-independent denaturing high performance liquid chromatography (DHPLC) to separate and identify PCR-amplified 16S rRNA sequences from genomic DNA purified from midstream urine specimens [316]. In this study of renal transplant recipients, a group at high risk for UTI. Interestingly, while the authors report 100% correspondence between culture-based method and detection of the corresponding *aerobic* bacteria in DHPLC, they also demonstrate that 9.2% of all patients examined were 'culture-negative' using standard approaches, yet appeared to contain significant numbers of fastidious bacteria detected using the DHPLC approach. Most of the culture-negative, DHPLC-positive specimens were polymicrobial, and many of the genera detected in these urines have been previously associated with BV (see above). Specifically, *Gardnerella vaginalis, Anaerococcus lactolyticus*, and *Lactobacillus iners* were among the Gram-positive bacteria detected in urines of renal transplant recipients and *Prevotella, Bacteroides, Dialister, Leptotrichia*, and *Fusobacteria* were among the Gram-negative bacteria detected. Many of these urines were positive for leukocyte esterase activity, strongly suggesting that the organisms present were the cause of symptomatic UTI as opposed to the possibility of urine contamination by vaginal bacteria. The authors state that standard curves of bacteria in pure culture were used to estimate CFU in urine using the DHPLC method and that in many cases bacteria were in clinically-relevant ranges of $>5\times10^4$ CFU/ml. Similar studies using midstream urine to assess bacterial diversity using 16S RNA sequencing have also implied that a variety of vaginal bacteria may inhabit the female urinary tract [318] [319]. One limitation of these studies is that the collection method (midstream "clean catch") does not adequately address the possibility of urine contamination with vaginal or periurethral microbes.

One line of evidence that microbes detected by culture-independent approaches are not simply a product of urine contamination by vaginal bacteria comes from a study in which bacteria recovered from catheters were identified by culture-dependent and -independent approaches [320]. As with previous studies, the authors demonstrate the presence of many bacteria that are currently regarded as vaginal bacteria (see Table 1). As with previous studies, examples of patients were provided in whom culture independent approaches detect high levels of bacteria such as *Actinobacculum* that were not amenable to culture using routine methods. The authors concluded that catheterized patients who had a UTI were more likely to have lower diversity of organisms recovered from catheters compared to patients who did not have a UTI. However, it is not clear from the methods what time point the 16S data contributing to this interpretation were collected (e.g. during UTI or prior to UTI). Thus, it is not clear if higher diversity of the urinary tract microbiota may protect against UTI or if the presence of a UTI drowns out the signal of other bacteria.

In an attempt to demonstrate beyond reasonable doubt that 'vaginal bacteria' in the urinary tract were in fact located in the bladder, a recent study by Wolfe et al., compared the bacterial composition of urine from asymptomatic adult women collected by various

methods including midstream 'clean catch,' transurethral catheterization, and direct suprapubic aspiration from the bladder [317]. Suprapubic aspiration eliminates concerns of vaginal or periurethral contamination. All urines used in the study were culture-negative using the clinical microbiology laboratory-recommended cutoff of 10^4 CFU/ml. The authors showed that a subpopulation of urines collected by transurethral catheterization did not yield significant growth under typical clinical microbiology laboratory conditions (for urine), but contained Gram-positive and Gram-negative bacteria that were evident by direct microscopy and 16S rRNA amplification and sequencing. Surprisingly, bacterial genomic DNA could be amplified from 21/23 urine samples collected by direct needle aspiration from the bladder. Organisms present in these specimens could often be observed in corresponding catheter and midstream specimens collected at the same time, but rarely from 'mock needle stick' samples. Bacteria detected in urine specimens obtained by catheterization or aspiration included the anaerobic genera *Actinobaculum, Anaerococcus, Atopobium, Gardnerella, Prevotella, Sneathia,* and *Veillonella* among others (see Table 1). These organisms are all fastidious anaerobes that would not be detected using typical aerobic urine culture and were not detected in mock needlesticks that did not enter the bladder. In addition to these somewhat unexpected organisms, more typical potential uropathogens were also revealed by this study, including species of *Streptococcus* and *Staphylococcus.*

These findings are supported by a few much earlier studies. For example, in a 1979 study. 185 pregnant women (admitted for pregnancy complications) underwent suprapubic bladder aspiration and their urine was subjected to anaerobic culture. 6.4% of these women had anaerobes in their urine including members of the genera *Peptostreptococcus, Veillonella, Clostridium*, and *Bifidobacterium* [321]. These findings provide clear evidence that urine taken directly from the bladder, and thus not exposed to potential contaminants of the vagina, periurethral area or distal urethra, often contains organisms that are undetectable using routine clinical microbiology procedures.

Independent investigations have also evaluated the microbial content of *male* urine or urethral swabs using culture-independent approaches. These studies reveal strikingly similar patterns of fastidious and anaerobic bacteria seen in female urogenital specimens, including more typical Gram-positive uropathogens (e.g. *Staphylococcus, Enterococcus, Streptococcus*), as well as other bacteria previously considered to be specific to the female urogenital tract (e.g. *Gardnerella, Atopobium, Actinobaculum, Anaerococcus, Lactobacillus*, etc, see Table 1) [322, 323]. Another recent study investigated the microbiota of urine and coronal sulcus specimens from adolescent males, showing that bacteria associated with BV are present in young men that report having *no history* of partnered sexual activity [324]. These data provide further evidence that specific bacterial species not cultivated using routine approaches are in fact present in the genitourinary tracts of both men and women and are not necessarily acquired through sexual contact. Note that some of these species have also been reported in case studies of urinary tract infection, most often in populations with underlying risk factors for UTI (see Table 1 references).

One of the major limitations of culture-independent urinary microbiome studies is that it is not possible to know whether the bacteria are alive or dead. A skeptic might ask"is the presence of microbial DNA in urine, even if taken by needle aspiration from the bladder,

indicative of a live microbial community?" Teams led by Schreckenberger, Wolfe, and Brubaker recently used an expanded quantitative urine culture (EQUC) method to address this important question [292, 325]. These authors demonstrated previously that urine collected from women by transurethral catheterization and by suprapubic aspiration had very similar microbiome profiles. Thus, subsequent studies used catheter-based collection of urine because this was the least invasive methods that still provided confidence that the urine was not contaminated with vaginal bacteria. The authors performed 16S rDNA-based community profiling in parallel with EQUC and demonstrated that many of the fastidious bacteria detected in catheterized urine by culture-independent approaches can in fact be recovered in laboratory culture [292, 325]. Whether these bacteria represent stable communities living in the bladder still remains to be fully characterized.

This same group most recently performed community profiling and EQUC on catheter-collected urine samples to test the hypothesis that the urinary microbiome may contribute to urinary urgency incontinence (UUI) [292]. Overall, this paper sets an impressive standard for the field, providing a truly multidisciplinary approach to provide evidence that challenges previous assumptions regarding a condition (UUI) of uncertain etiology that has nevertheless been defined as noninfectious. The authors conclude that the urinary microbiomes of women with UUI were in fact different from women in the control group, having lesser proportions of *Lactobacilli* and greater proportions of *Gardnerella, Actinobacculum, Actinomyces, Aerococcus, Arthrobacter, Corynebacterium, Oligella, Staphylococcus*, and *Streptococcus*. This paper also reports that women in the UUI group were more likely to harbor *Lactobacillus gasseri* in their bladders compared to women in the control group, which were more likely to harbor *Lactobacillus crispatus*. In addition to the culture independent component of this study, the authors also performed EQUC to demonstrate that many of the bacteria detected using molecular approaches could also be cultured in the laboratory. One potential limitation of this study is that the UUI group was found to be ~2-fold less likely to be using estrogen, despite being on average 14 years older than the control group (63 [±12] versus 49 [±14]; P < 0.05). Differences in age and hormonal status have a wide variety of physiological implications and have been correlated with specific phenotypic features of the vaginal microbiota (e.g. presence or amounts of various bacteria) [326, 327]. These factors could therefore be important confounders that may contribute to the differences observed between the cases and controls.

Another study from the same groups used the same population of women suffering from UUI who were randomized to two treatment arms of a large clinical trial, but instead employed qPCR targeting the 16S ribosomal DNA (rDNA) to assign women simply as positive or negative for bacteria presence. Only women who were negative for UTI by the clinical (culture-based) definition at the time of urine sample retrieval, prior to randomization were included in the qPCR study. The authors report no statistically significant differences in median qPCR levels depending on whether women went on to have a post procedure UTI (UTI 2.58×10^5 vs no UTI 1.35×10^5 copies/ml) or whether treatment for UUI was successful. Rather, using a categorical variable (presence/absence), the authors demonstrate that women positive for bacterial DNA in catheterized urine suffered a higher number of UUI episodes (5.71[±2.60] vs 4.72 [±2.86]). However, qPCR positive women

were less likely to suffer from post-procedure UTI compared to qPCR negative women (10% vs 24% respectively).

One of the limitations of the urinary tract microbiome studies performed to date is that the abundance and composition of microbes in the urinary tract is relative and based on the total number of sequence reads, which can vary widely from individual to individual. Going forward, one of the challenges will be to measure absolute abundances of specific uncultivated species in larger epidemiological studies, and to evaluate the potential risks posed by urinary tract colonization with different bacterial species or subsets. Experimental models in animals should also be employed to help to directly examine the hypothesis that some of these Gram-positive anaerobes are pathogenic in the urinary tract. Such models will also be required to test specific hypotheses related to the potential roles of urogenital bacteria in determining host susceptibility to UTI caused by common uropathogens.

Many of the fastidious anaerobes described in urine microbiome studies (see Table 1) are taxa that have also been associated with bacterial vaginosis. Clinical studies demonstrating a higher risk of UTI in women with BV further suggest that some of these bacteria may simultaneously cause dysbiosis in the vagina and the bladder. However, contamination of urine with vaginal bacteria is not a viable explanation for these findings given that the bacteria were also detected in urines collected by catheterization and suprapubic aspiration (see Table 1). As described above, the presence of clue cells (i.e. squamous epithelial cells covered in *G. vaginalis* and possibly other bacteria) is characteristic of women with BV [222]. However, consistent with the idea of urinary tract dysbiosis, women with "sterile pyelonephritis" who were identified as having a high infectious bladder burden of *G. vaginalis* also had clue cells in urine directly aspirated from the bladder [291]. In fact, clue cells have also been reported in urethral and semen specimens from men who were identified as having *G. vaginalis*-associated infections [307–309]. These findings support the interpretation that *G. vaginalis* may attach itself to epithelial cells of the urethra and bladder and induce exfoliation, thus resulting in urinary clue cells that much resemble the vaginal clue cells seen in women with BV.

Experimental studies are needed to determine whether these 'BV bacteria' have a direct causal role in determining host susceptibility to acute or recurrent UTI. Findings that women suffering from recurrent UTI have fewer recurrences following vaginal probiotic *L. crispatus* administration suggest that vaginal lactobacilli may have a protective role in UTI, or that they may displace other organisms that have a detrimental role. This interpretation is also indirectly supported by observations that *Lactobacillus* is the most common dominant organism in the urinary tract (among older women) and that having qPCR-positive urine was associated with a reduced likelihood of UTI (see above). Finally, the fact that these bacteria can also be found in the male urinary tract, even among adolescent males with no sexual history, strongly suggests that these 'BV bacteria' are not simply vaginal bacteria transiently exposed to the urinary tract during sexual activities, but rather that these organisms may have a wider tissue tropism that includes the urinary tract.

In summary, we have 1) reviewed the epidemiology and virulence characteristics of the most common Gram-positive uropathogens, including *Staphylococcus saprophyticus,*

Enterococcus faecalis, and group B *Streptococcus*, 2) reviewed the natural history of several less common uropathogens and the reasons why these bacteria are systematically overlooked, 3) summarized recent literature suggesting that a wide variety of Gram-positive bacteria are common inhabitants of the human urinary tract, and 4) presented evidence that polymicrobial infections involving Gram-positive bacteria may be more prevalent than previously appreciated and also may impact pathologic outcomes in the urinary tract.

Fifty years ago it was thought that the major uropathogenic organisms did not include Gram-positive bacteria. Ten years ago it was assumed that the uninfected urinary tract was sterile. Currently, it is widely held that Gram-positive bacteria either alone or alongside Gram-negative uropathogens in the urine are likely to be contaminants of no consequence. The first two assumptions have been disproved as technology has advanced. The literature summarized in this chapter calls into question the last assumption. We propose that future studies will illuminate a previously unappreciated role for members of the polymicrobial microbiota in the urinary tract, vagina, and gut in UTI susceptibility and disease progression.

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Describes *Staphylococcus, Enterococcus*, and *Streptococcus* as uropathogens, discusses polymicrobial urinary tract infection, the significance of polymicrobial growth upon urine culture, and the potential under-reporting of fastidious organisms and atypical Gram-positives as causes of urinary tract infection. Finally, this chapter summarizes current literature regarding the microbiota of the urinary tract and discusses the potential role of urogenital microbiota in UTI susceptibility.
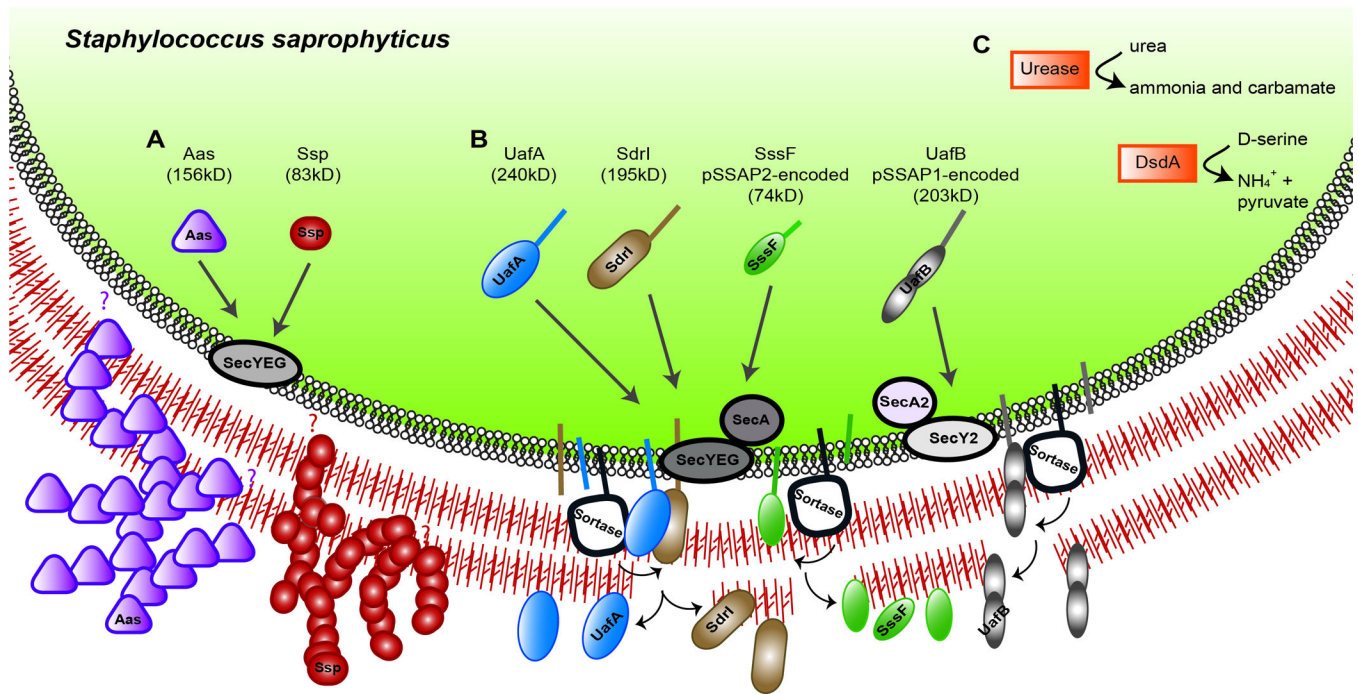
**Figure 1. *S. saprophyticus* virulence factors**

(A) Secreted surface proteins: Aas, possesses N-terminal signal sequence, but no motifs such as a transmembrane domain, LPXTG sortase recognition motif, or proline/glycine-rich cell wall-spanning domain to indicate the mode of attachment to the cell surface after translocation across the membrane (indicated by the purple question marks) [72]. Immuno-elctron microscopy shows Aas as part of a fuzzy surface layer that is absent when Aas is not expressed [75]. Ssp has a YSIRK-containing signal sequence but no sortase-recognition motif so its mode of attachment to the cell surface is uncertain (indicated by the red question mark); it is easily sheared from the cell surface. Electron microscopy and immuno-electron micrographs also show Ssp to exist as part of fuzzy surface layer, apparently consisting of 50–75nm fibrillar structures; the nature of these fibers in not known [75, 76]. (B) UafA, SdrI, SssF, and UafB contain an LPXTG motif and are predicted to be covalently attached to the cell wall [78, 79, 81]. The small arrows near the membrane-anchored sortase enzymes indicate the two-step transpeptidation reaction whereby sortase substrate are first cleaved within the LPXTG motif to create a sortase-substrate intermediate (and releasing the membrane domain and positively charged cytoplasmic tail, indicated by the straight line in the membrane) that is then resolved resulting in covalent linkage of the substrate to the cell wall [380]. UafB is genetically linked to accessory secretion genes *secA2* and *secY2* that are predicted to encode a dedicated accessory secretion system for UafB [79]. (C) Cytoplasmic enzymes that promote *S saprophyticus* survival in urine [381, 382].
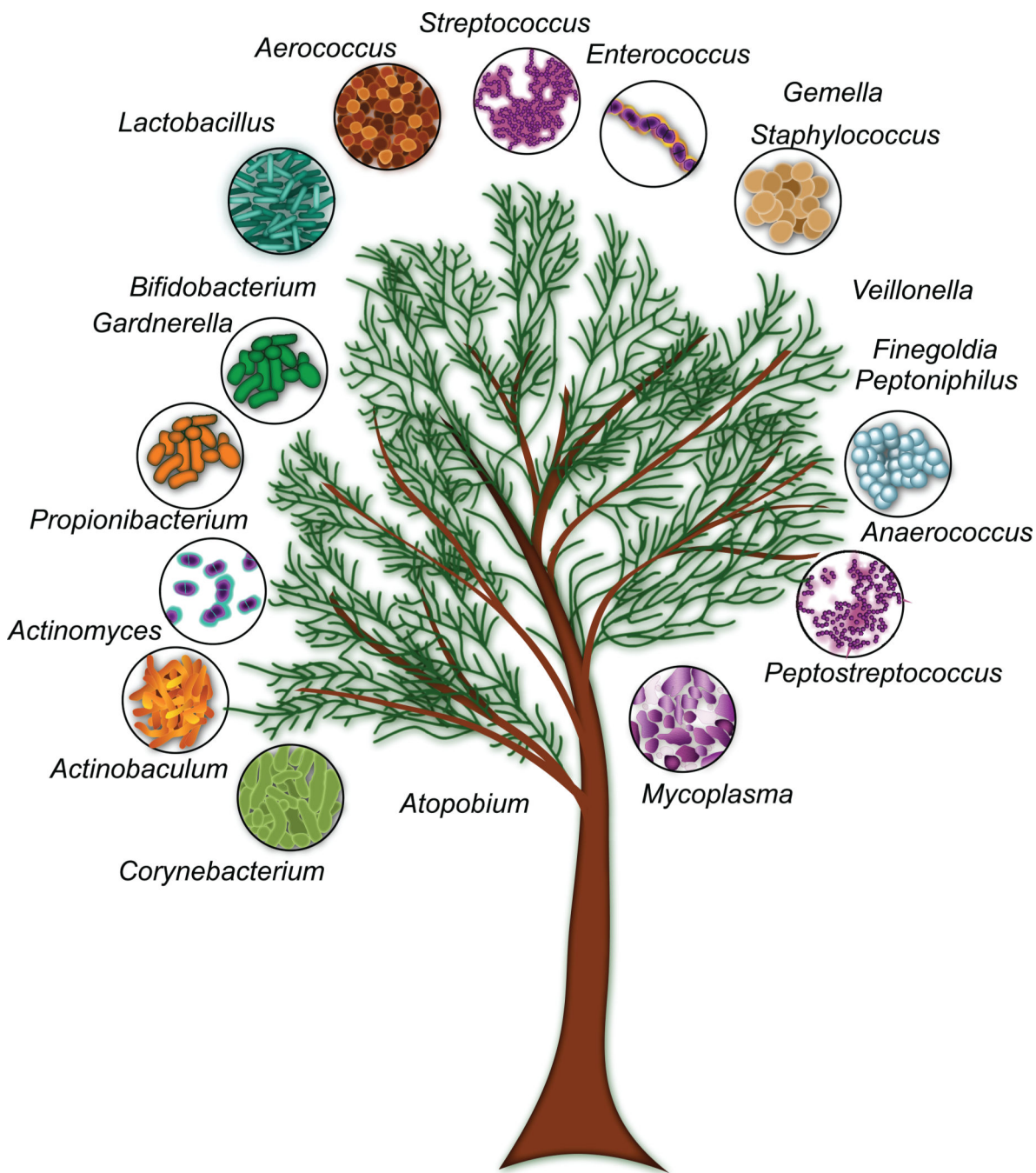
Figure 2. Gram-positive inhabitants and pathogens of the human urinary tract

Approximate phylogenetic relationships between Gram-positive bacteria are illustrated in this schematic representation. Please refer to the text and Table 1 for additional information and references describing these genera as uropathogens or inhabitants of the human urinary tract. On the left, *Bifidobacterium* and *Gardnerella* belong to the order *Bifidobacteriales,* which together with the orders *Priopionibacteriales, Actinomycetales, and Corynebacteriales* belong to the class *Actinobacteria* (a.k.a. "high GC Gram positive bacteria"). *Atopobium* belongs to the order *Coriobacteriales* and the class *Coriobacteriia*. The classes *Coriobacteriia*

and *Actinobacteria* both belong to the phylum *Actinobacteria*. The remaining genera with the exception of *Mycoplasma* belong to the phylum *Firmicutes.* Whereas *Peptostreptococcus, Anerococcus, Finegoldia* and *Peptoniphilus* belong to the order *Clostridiales* and the class *Clostridia, Staphylococcus* and *Gemella* belong to the order *Bacilliales* and the class *Bacilli*. Members of the order *Lactobacilliales* (*Lactobacillus, Aerococcus, Enterococcus,* and *Streptococcus*), are also classified as *Bacilli*. The genus *Veillonella* is also a member of the *Firmicutes*, but belongs to the class and order *Negativicutes* and *Selenomonodales* respectively. On the other hand, *Mycoplasma* belongs to the phylum *Tenericutes*, the class *Mollicutes* and the order *Mycoplasmatales*.
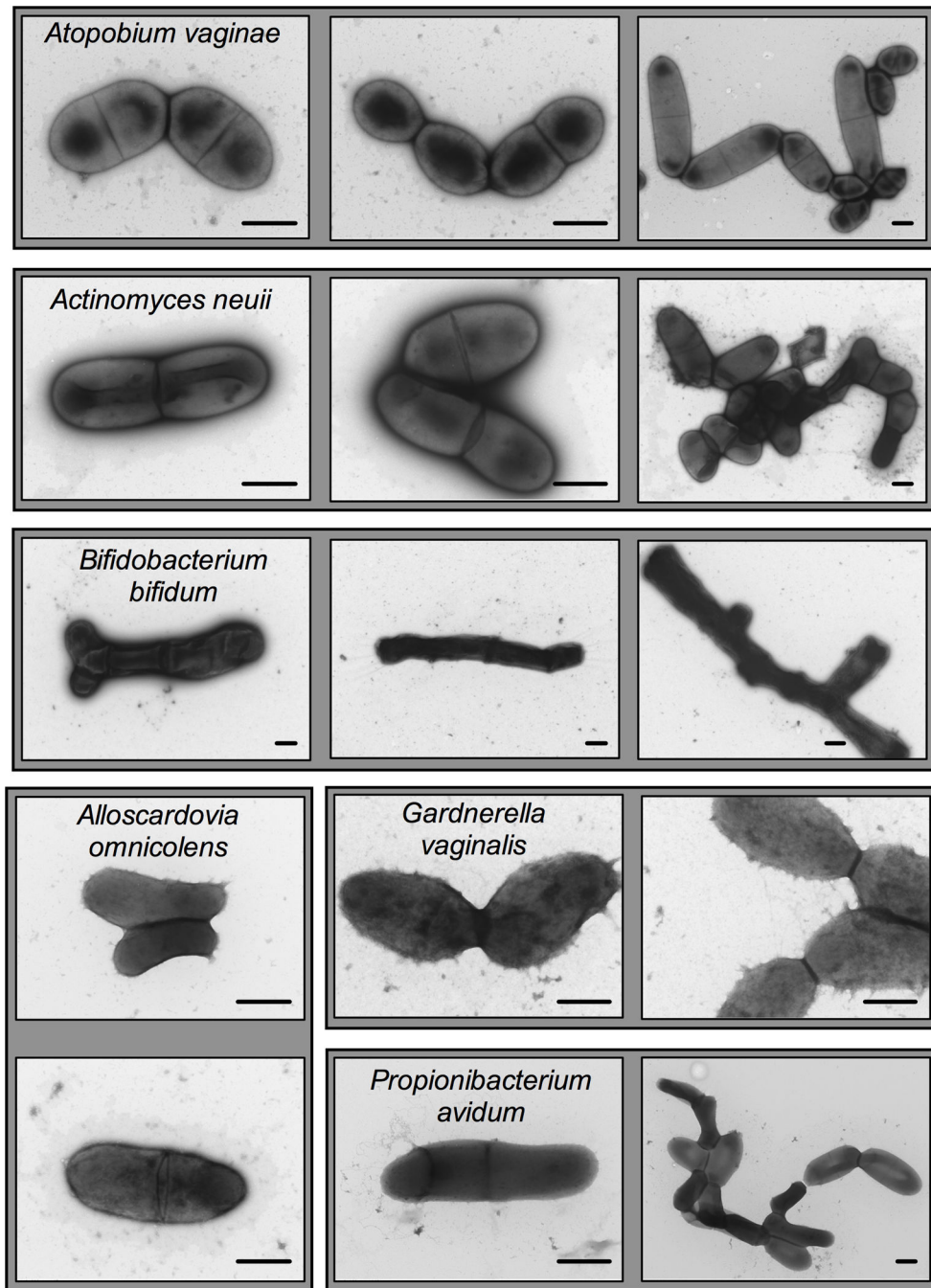
Figure 3. Transmission electron micrographs of several urogenital isolates from the phylum *Actinobacteria*

Strains grown for 24–48 hours underwent negative staining with uranyl acetate and were examined by TEM. These strains were isolated from the urine or vaginas of pregnant or nonpregnant women and are available through BEI resources. Strain names are as follows: *Actinomyces neuii,* MJR8396A; *Alloscardovia omnicolens,* CMW7705A; *Atopobium vaginae,* CMW7778A; *Bifidobacterium bifidum,* MJR8628B; Gardnerella vaginalis,

PSS7772B; Propionibacterium avidum, MJR7694. Scale bars are 500nm. Shaded backgrounds contain images of the same strain.

Table 1

Genera of Gram-positive human urinary tract inhabitants and pathogens detected by culture-dependent and –independent techniques

"culture negative" means specimens that did not reach the cutoff value, usually 10^5 CFU/ml urine in a clean catch specimen or 10^4 CFU/ml for collections by catheter or suprapubic aspiration.

Phylum	Genus	Culture-Dependent Clinical Reports			Culture-Independent Detection in Urinary Tract								
		Bladder/ Kidney infection	Bacteremia & Urosepsis	Urethritis	Female, culture unknown	Female, culture positive	Female culture-negative, nitrite and leukocyte esterase negative or asymptomatic			Male asymptomatic		Male symptomatic
					TUC	CC,TUC	CC	TUC	SPA	urine	urethral swab	urine
	Staphylococcus	5–20% of CAUTI [47]	[328]	[53, 55, 56]	[292, 329]	n/r	[318, 319]	[317, 329]	[317]	[319, 322–324]	[323]	[330]
	Enterococcus	15–30% of CAUTI [13, 115, 119]	[328]	[331, 332]	[318]	[316, 333]	[319]	n/r	n/r	[322, 323]	[323]	[330]
	Streptococcus	1–2% of UTI [180]	[180]	[334]	[292, 318, 329]	n/r	[316, 318, 319]	[317, 329]	[317]	[322–324]	[323]	[330]
Firmicutes	Aerococcus	[230, 232, 237, 239]	[233–236, 278]	n/r	[329, 335]	[317]	[318, 319]	[317]	[317]	[319, 322, 323]	[323]	n/r
	Anaerococcus	n/r	n/r	n/r	[292, 329, 335]	[333]	[316, 318, 319, 333]	n/r	[317]	[319, 322–324]	[323]	n/r
	Peptostreptococcus	[321, 336]*	[337]	n/r	[335]	[333]	[318, 319]	n/r	n/r	[319, 322, 324]	n/r	n/r
	Peptoniphilus	n/r	[338]	n/r	[335]	[333]	[317–319]	n/r	n/r	[319, 322]	n/r	n/r
	Lactobacillus	[321, 339, 340]*	n/r	n/r	[335]	[333]	[316, 319, 333]	[317, 329]	[317]	[319, 322–324]	[323]	[330]

Phylum	Genus	Culture-Dependent Clinical Reports			Culture-Independent Detection in Urinary Tract								
		Bladder/ Kidney infection	Bacteremia & Urosepsis	Urethritis	Female, culture unknown	Female, culture positive	Female culture-negative, nitrite and leukocyte esterase negative or asymptomatic			Male asymptomatic		Male symptomatic
					TUC	CC,TUC	CC	TUC	SPA	urine	urethral swab	urine
	Finegoldia	n/r	n/r	n/r	[335]	[317, 318, 333]	[319]	n/r	[317]	[319, 322_324]	[323]	[330]
	Veillonella	[341, 342]	[337, 341]	n/r	[335]	[317]	n/r	[292, 329]	n/r	[322, 323]	[324]	n/r
	Genella	[343]	n/r	n/r	n/r	n/r	n/r	n/r	n/r	[319, 322, 323]	[323]	n/r
	Corynebacterium	[246_249, 344_349]	[350]	[351_353]	[292, 329, 335]	n/r	[316, 318, 319]	[317, 329]	[317]	[319, 322, 323]	[323]	n/r
	Actinobaculum	[155, 261, 262, 266, 267, 269, 272, 274, 276, 277, 354]	[270, 275, 278, 355]	n/r	[335]	[317, 333]	[318, 319]	[292, 317, 329]	[317]	[322]	n/r	n/r
Actinobacteria	Gardnerella	[285, 300]*	[293, 300, 303, 356]	[307_309, 357_359]	[335]	[333]	[316, 318, 319, 333]	[317, 322_324, 329, 360]	[317]	[319, 322]	[323]	n/r
	Atopobium	n/r	n/r	n/r	[335]	[333]	[317, 319, 333]	[292, 317, 329]	[317]	[319, 322, 323, 361]	[323]	[361]
	Actinomyces	[362_366]	[367]	n/r	[335]	n/r	[319]	[292]	n/r	[322]	n/r	n/r
	Bifidobacterium	n/r	n/r	n/r	[335]	[333]	n/r	[292, 329]	n/r	n/r	n/r	[330]
	Mycoplasma	[368_371]	n/r	[358, 372_379]	n/r	n/r	n/r	n/r	n/r	[319, 322, 323]	[323]	n/r

n/r = not reported, CAUTI = community acquired UTI,

* Questionable clinical significance, CC = clean catch, TUC = transurethral catheter, SPA = suprapubic aspiration.