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Defining a Molecular Signature for Uropathogenic *versus* Urocolonizing *Escherichia coli*: The Status of the Field and New Clinical Opportunities

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Abstract

Urinary tract infections (UTIs) represent a major burden across the population, although key facets of their pathophysiology and host interaction remain unclear. *Escherichia coli* epitomizes these

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmb.2019.11.008>.

obstacles: this gram-negative bacterial species is the most prevalent agent of UTIs worldwide and can also colonize the urogenital tract in a phenomenon known as asymptomatic bacteriuria (ASB). Unfortunately, at the level of the individual *E. coli* strains, the relationship between UTI and ASB is poorly defined, confounding our understanding of microbial pathogenesis and strategies for clinical management. Unlike diarrheagenic pathotypes of *E. coli*, the definition of uropathogenic *E. coli* (UPEC) remains phenomenologic, without conserved phenotypes and known genetic determinants that rigorously distinguish UTI- and ASB-associated strains. This article provides a cross-disciplinary review of the current issues from interrelated mechanistic and diagnostic perspectives and describes new opportunities by which clinical resources can be leveraged to overcome molecular challenges. Specifically, we present our work harnessing a large collection of patient-derived isolates to identify features that do (and do not) distinguish UTI- from ASB-associated *E. coli* strains. Analyses of biofilm formation, previously reported to be higher in ASB strains, revealed extensive phenotypic heterogeneity that did not correlate with symptomatology. However, metabolomic experiments revealed distinct signatures between ASB and cystitis isolates, including in the purine pathway (previously shown to be critical for intracellular survival during acute infection). Together, these studies demonstrate how large-scale, wild-type approaches can help dissect the physiology of colonization *versus* infection, suggesting that the molecular definition of UPEC may rest at the level of global bacterial metabolism.

Keywords

Asymptomatic bacteriuria; Cystitis; Metabolomics; Urinary tract infection; Uropathogenic *Escherichia coli*

Introduction

UTI and ASB: a complex clinical epidemiology

From research laboratories to intensive care units, bacterial urinary tract infections (UTIs) are an increasingly complex healthcare challenge. Overall, UTIs account for approximately 20 million ambulatory visits annually across the United States and Europe [1–3], and it is estimated that at least fifty percent of all women experience at least one UTI during their lifetime [1,2,4]. If left unchecked, bladder infections (cystitis) can progress to the kidneys (pyelonephritis) or prostate (prostatitis), with the potential for bloodstream infection (bacteremia) and life-threatening sepsis (Fig. 1a and [5,6]). In functional parlance, UTIs are typically designated as either uncomplicated or complicated, with the latter often defined broadly as an infection in an individual with some structural, functional, or immunologic predisposition to urologic infection [7].

Conversely, in the common phenomenon of asymptomatic bacteriuria (ASB) (Fig. 1a), the same species that cause infections are cultured from the urine of healthy patients without any associated signs or symptoms [8–14]. ASB is defined in the context of one or more bacterial species isolated at high-quantitative burden (>100,000 CFU/mL) from traditional, clean-catch diagnostic cultures (see below for additional discussion on what constitutes such an assay) [15]. Some criteria for ASB stipulate the same organism being isolated from repetitive samples in asymptomatic patients [16], although in clinical practice a single urine

specimen is the diagnostic norm. The prevalence of ASB increases with age, with rates in elderly women of up to ~20% (compared with <5% in healthy premenopausal subjects); other risk factors for ASB include long-term admission to a healthcare facility, along with many of the same predisposing structural or functional factors as complicated UTIs (as previously reviewed [15]). Serving as a central theme to the current manuscript, a prominent hurdle to preventing and managing UTIs is the inability to distinguish—both biologically and diagnostically—bacterial strains associated with symptomatic infections from those that cause ASB.

The ability to discern ASB from true UTIs is particularly challenging in the context of subtle or ambiguous symptoms, which further complicate administering the appropriate course of medical management. Although current guidelines do not recommend treatment for most patients with ASB [15], the same is not universally true for pregnant women [17], individuals with spinal cord injury [18], bladder cancer [19], and renal allograft recipients, for whom antibiotics are commonly administered [20]. This concern for unnecessary treatment of ASB stems from the rapidly evolving epidemiology of antimicrobial resistance (in the urologic context and broadly) and the recognition of antimicrobial stewardship as an important public health priority [21,22]. Critically, not only do UTIs frequently occur among both healthy and debilitated patients, but resistant strains are increasingly encountered [1,4–6,23,24]. For example, the prevalence of beta-lactamase encoding *Enterobacteriaceae* has required updated guidelines on what agents constitute appropriate empiric therapy for uncomplicated UTIs (removing traditional penicillins) [25]. Going further, the threat of multidrug-resistant (MDR) strains looms large, including the emergence of the MDR ST131 lineage in *E. coli*, which is resistant to extended spectrum cephalosporins and fluoroquinolones. In 2017, the World Health Organization added carbapenem-resistant and ESBL-producing *Enterobacteriaceae* to their Critical Priority List for which new antibiotics are needed [26].

The lifestyle of *Escherichia coli* as a uropathogen and urocolonizer

Among the causes of UTIs, *E. coli* is the most prevalent species worldwide [1,4], a trend that is reflected by local data at Vanderbilt University Medical Center (VUMC) (Fig. 1b). In addition to causing frank infection, *E. coli* is also a common agent of ASB across diverse groups [27]. Although the taxonomy of ASB-associated organisms tends to be more diverse and variable than symptomatic UTIs, *E. coli* remains among the most prevalent species (often the most prevalent) observed in different populations, including patients with spinal cord injuries [28–30], urethral catheters [28,31,32], ureteral stents [33,34], and those admitted to long-term care facilities [28,35]. At the same time, despite high symptomatic and asymptomatic prevalence, we lack empiric metrics—at the level of individual *E. coli* isolates—to judge whether a strain is uropathogenic or merely urocolonizing. The classification of UPEC as a discrete pathotype differs from strains of *E. coli* that elicit diarrheal illness (e.g. enterotoxigenic/ETEC or enterohemorrhagic/EHEC). Although these enteric pathotypes can be identified pathogenetically and diagnostically by the presence of discrete genetic virulence determinants—such as Shiga-like toxin, heat labile/stable toxin, the type-3 secretion apparatus, and components of attaching/effacing lesions [36–40]—UPEC does not carry a rigorous molecular definition [41,42]. In fact, the term “UPEC”

remains phenomenologic, simply denoting strains isolated from cases of symptomatic UTI. What remains imprecise in this context is the classification and ontogeny of ASB-associated strains. A comparison of “classic” monogenic virulence factors between UTI- and ASB-associated strains reveals significant genotypic and phenotypic overlap, with no single bacterial marker distinguishing uropathogens from urocolonizers [41–43]. For example, all UPEC isolates carry at least one iron acquisition system (with some encoding six or more), but only about fifty percent of the isolates harbor the hemolysin toxin [44–47]. Similarly, though type 1 pili (*fim*) are critical for infection establishment by UPEC strains, the *fim* locus is harbored by the vast majority of *E. coli* strains sequenced to date and are not specific to UPEC.

On a phenotypic level, previous studies have suggested that ASB isolates may elaborate higher levels of extracellular biofilm, thereby protecting the host through competitive exclusion of uropathogenic strains [48–51]. For this procedure, antibiotics are administered before the instillation of the ASB strain to sterilize the bladder. After an antibiotic-free interval, the patient is catheterized, the bladder is emptied, and 30 mL *E. coli* 83972 (10^5 CFU/mL) injected. This procedure is repeated daily for up to three days until bacteriuria is established [49]. Although strain 83972 has been deemed the “prototypical” ASB isolate and has been widely characterized and now promoted for probiotic use [14,49], there remain extensive gaps in our ability to fully understand the molecular basis that distinguishes ASB from true UPEC isolates. Furthermore, we do not know if there distinguishing differences in the metabolic capacity of ASB isolates that can be leveraged for the development of diagnostic strategies. Previous reports described metabolic requirements for UPEC during acute UTI in an experimental murine model of infection, identifying the need for amino acid utilization, the TCA cycle and aerobic respiration, as well as *de novo* purine synthesis [52–55]. How extensively these requirements overlap with ASB isolates remain unknown.

Commensurate challenges in pathogenesis and diagnosis

The uncertainties surrounding the pathogenesis of UPEC and its relationship to ASB are reflected (and in some cases driven) by corresponding challenges in the diagnostic setting. The standard approach for diagnosing UTIs in clinical laboratories involves semi-quantitative urine culture of symptomatic patients [56–58], with higher colony counts of a single organism (*i.e.* >100,000 CFU/mL) suggesting a causative relationship, as opposed to periurethral contamination (for a poorly collected, non-mid-stream specimen). Recent studies have challenged this paradigm, however, demonstrating that symptomatic UTIs can arise with low quantitative burdens of *E. coli* (and other organisms) in urine [59], sometimes even at levels (<1000 CFU/mL) below the threshold of typical clinical detectability (given a standard 1 μ L inoculum). This observation may come as unsurprising to investigators of UPEC pathogenesis, given the ability of *E. coli* to persist invasively within urothelial cells [60], but it represents an extremely challenging reality for the workflows and logistical capabilities of diagnostic laboratories. Importantly, it creates an even greater overlap between bacterial burdens that may be observed in UTI and ASB (given a properly collected midstream or catheterized specimen) or even just periurethral contamination. From a clinical perspective, the ability to differentiate these scenarios and selecting if (and with what agent) to treat becomes even more complicated.

The current lack of a rigorous molecular definition UPEC has likewise prevented UTI management from entering the burgeoning era of molecular diagnostics, at least at commensurate levels to other infections and anatomic sites. It is instructive to consider the dynamic within the human gut, where strains of *E. coli* are essentially ubiquitous but certain pathotypes are associated with diarrheal illness. As aforementioned, these pathotypes are defined by genetic determinants that not only facilitate host interaction and symptomatic infection but can be detected as discrete targets by nucleic acid amplification techniques. This has made possible the development of culture-free diagnostic assays to detect pathogenic strains, despite the background presence of commensal *E. coli*. In particular, syndromic molecular testing has grown in clinical popularity, in which multiplex PCR assays are used to identify at once a number of causative pathogens within stool (and other specimen types), including multiple enteric pathotypes of *E. coli* [40]. Commercially available assays now exist that identify, for instance, Shiga-toxin producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli*/Shigella (EIEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* [38,61–64]. However, diagnostic financial considerations aside, a molecular assay for identifying UPEC UTIs would be hampered physiologically and genetically by the prevalence of ASB and our inability to differentiate ASB and UTI strains. If a genetic definition of UPEC does exist, it does not appear to be at a simple monogenic level, limiting an ability to develop diagnostic sets of primers and probes.

Finally, an additional source of historic confusion stems from the practice of culturing urine specimens under growth conditions tailored to identify major pathogenic species (*i.e.* with an aerobic atmosphere, on standard bacteriologic agars, and with short incubation times). Because of these methods, it was long assumed that the human bladder is physiologically sterile. This viewpoint has now been disproven, as enhanced culture-based and molecular techniques have demonstrated physiologic colonization of the bladder by diverse and often fastidious bacterial species [65–73]. In fact, one could argue that the clinical definition of ASB should be amended in light of this urinary microbiome, entailing the isolation of organisms from the urine of asymptomatic patients, but using traditional, unenhanced microbiologic techniques. If diagnostic urine specimens were routinely cultured for protracted times under alternative atmospheric conditions, the prevalence of ASB would undoubtedly be far higher, especially at lower organism burdens. In medical practice, unfortunately, physicians are faced with formidable challenges in integrating such discoveries with evidence-based interpretation of clinical laboratory results. Even seemingly clear-cut guidelines such as *do not treat ASB with antibiotics* are frequently obscured by challenging clinical scenarios. Common within many high-risk settings are patients with objective bacteriuria, but whose clinical pictures are ambiguous or have alternate potential etiologies for their signs and symptoms. For precisely these women and men, an empiric diagnostic test to gauge whether an *E. coli* strain is pathogenic or merely colonizing would carry high value for prognosis and management.

New opportunities for translational discovery

Fundamentally, the abovementioned challenges surrounding *E. coli* and UTI/ASB are a direct function of high biologic diversity: just as the clinical phenotypes and symptomology of bacteriuric patients are incredibly heterogeneous, so too is the genotypic and phenotypic

landscape of the responsible strains. To define subtle underlying relationships between host and microbe, this real-world complexity may require both of these components to be networked in tandem, across individual clinical cases. Unfortunately, such a paradigm is not always compatible with traditional molecular strategies, in which mechanistic questions are dissected through model organisms and experimental systems. Despite these challenges, one notable resource is the sheer prevalence of UTI and ASB across the population and the volume of cases that are diagnosed via microbiologic culture by clinical laboratories at large medical centers, with the resultant bacterial isolates remaining as the routine derivatives of care. Although they are typically discarded after diagnosis, we hypothesized these organisms could serve as wild-type resources for discerning organismal features in *E. coli* that differentiate UTI- and ASB-associated strains, especially when paired with clinical data from source patients. In fact, if such features could be defined rigorously—at the genotypic, phenotypic, or some other *-omic* level—they would represent a *de facto* definition of “UPEC” itself.

In this light, our group has recently strived to harness the power of numbers inherent to the diagnostic setting, by establishing a microbial biobanking initiative—*microVU*—through which clinical isolates (derivatives of clinical care) are stored along with linked, yet deidentified, patient data for use in basic science research. Leveraging these resources, we conducted a pilot retrospective analysis of phenotypic and metabolic features of *E. coli* isolated from uncomplicated cystitis or cases of ASB. The following sections detail the findings of these analyses, which indicate that: (1) There is extensive phenotypic heterogeneity in both ASB and cystitis groups that prevents the reliable use of assays such as crystal violet—based biofilm determination for distinguishing ASB from UTI strains. This finding is in agreement with previous comparative studies among some model ASB and UTI isolates [14]. (2) None of the phenotypic analyses tested clearly segregate by patient symptomatology. (3) Large-scale, unbiased metabolomic analysis of ASB- and cystitis-associated strains grown in human urine revealed a distinct metabolic signature for cystitis strains, with purine metabolism—previously reported as critical for UPEC pathogenesis [55]—as one of the distinguishing pathways. In total, this work opens potential avenues for classifying UTI-versus-ASB strains of *E. coli*, providing novel metabolomic perspectives for the classification of UPEC. In the process, it highlights the value of large-scale, host-pathogen association studies in identifying molecular phenomenon that may escape traditional model systems.

Results

Study design and clinical epidemiology of urinary *E. coli*

To investigate whether a phenotypic or molecular signature can distinguish ASB- from UTI-associated strains of *E. coli*, a retrospective, single-site study was performed on 303 clinical isolates collected over the course of 4 weeks. These isolates correspond to cases where *E. coli* was isolated by the Clinical Microbiology Laboratory in high abundance (>100,000 CFU/mL) and monoculture (no coisolated organisms). For the purposes of this study, each strain was assigned the designation “VUTI” for Vanderbilt Urinary Tract Isolate, followed by a deidentified strain number.

In parallel, clinical phenotypes of each source patient were mined from the electronic health records (EHR) by the Clinical Laboratory Medical Director. Within the EHR (Epic Hyperspace), the following information sources associated with the particular culture were reviewed in detail: the *History & Physical* and *Progress* notes (for inpatients and individuals in the Emergency Department) and *Clinic Notes* and review-of-symptom questionnaires (for outpatients). Documentation of any of the following signs/symptoms—reported to or elicited by providers in the context of the patient’s presentation—qualified their *E. coli* strain as a UTI-associated: increased frequency or urgency; dysuria; lower abdominal pain, pressure, or other discomfort; or gross new-onset pyuria or hematuria. Acute altered mental status was also considered a subjective finding of UTI, so long as it was ultimately and specifically attributed as such by providers. Objectively, the documented presence of any SIRS criteria (fever, tachypnea, tachycardia, leukocytosis) also qualified a strain as UTI-associated. Urinalysis values were not utilized in themselves to designate an organism as such. Conversely, if none of the abovementioned criteria applied (and relevant urologic findings were actually included in the clinical notes), the *E. coli* isolate was classified as ASB-associated. For whatever reason, if the EHR did not include enough documentation describing the patient’s relevant status at the time of urine culture, the isolate was excluded from consideration altogether. From the 303 collected isolates, 18 were excluded from consideration (4 collected in error; 14 unclear). The remaining 285 strains were classified as UTI-associated ($n = 199$, 70.4%) or ASB-associated ($n = 86$, 29.6%) and analyzed in subsequent assays (Fig. 2). As expected, based on previous epidemiologic data, 247 out of the 285 strains were isolated from women (86.7%, Fig. 2), underscoring the prevalence of UTIs in females.

For both UTI- and ASB-associated isolates, the following demographic parameters and clinical phenotypes were abstracted as part of this chart review: legal sex; age range (18–35, 36–50, 50–60, 61–70, 71–80, 81+); collection setting (outpatient, inpatient, emergency department); ascending infection status (pyelonephritis—as explicitly diagnosed by the provider, bacteremia—as indicated by the concomitant isolation of *E. coli* from blood cultures); predefined risk factors (pregnancy, diabetes); and other structural or functional urinary tract abnormalities (also refer to accompanying Data in brief article, data worksheet 1). At this point, both the cultured organism itself and the corresponding list of annotations were permanently deidentified with the same VUTI number (per IRB protocol), before further investigational analyses for this study. The complete set of deidentified clinical parameters associated with each of the 285 isolates analyzed is provided in the accompanying data-in-brief article.

Biofilm phenotypes do not correlate with clinical infection scenarios

Among the 199 UTI-associated isolates, the majority were associated with a clinical picture of cystitis (176/199, 61.7%), with 19 (6.7%) and 4 (1.4%) isolates associated with pyelonephritis and bacteremia, respectively (Fig. 3a). Notably, there were also 5 isolates from the abovementioned groups (4 from cystitis cases and 1 from ASB) which were also associated with the presence of a catheter (please see Fig. S1a and accompanying Data in brief, data worksheets 2–3). To determine whether isolates associated with a specific disease state share common phenotypic features—that could potentially be useful in a high-

throughput setting such as the clinical lab—we evaluated both biofilm formation in liquid growth conditions (Fig. 3b), as well as colony morphology on nutrient agar (Fig. 4).

Biofilm levels of the 285 VUTI strains were measured using the crystal violet colorimetric assay of O'Toole [74], as previously described [48]. For the colony morphology studies, individual strains are spotted on Yeast Extract/Casamino Acid nutrient agar supplemented with the Congo red (CR) dye. Colonies appear red if there is extensive production of amyloid fibers (curli) or cellulose, the latter also associated with distinct visual rugosity (Fig. 4 and [75–77]). The resultant colonies were assessed for rugosity, CR uptake, as well as phenotypic instability, recorded as the presence of emergent subpopulations (Fig. 4). These emergent subpopulations typically remain stable on subculture (or semi-stable/unstable with occasional revertants or further variants; recorded in accompanying Data in brief, data worksheet 3), likely reflecting the emergence of genome-level variability [78].

Both the biofilm assays as well as the colony morphology assessments revealed extensive heterogeneity in all phenotypes tested, which did not significantly differ in ASB isolates (Figs. 3 and 4, S1b). This heterogeneity *in vitro* underscores these organisms' dynamic nature and the potential for commensurate diversity *in situ* (*i.e.* in the host), on top of the already considerable heterogeneity between strain backgrounds. Moreover, these large-scale phenotypic analyses indicate that phenotypic profiling on nutrient agar cannot be used as a distinguishing tool to separate ASB isolates from other urinary *E. coli* isolates.

ASB isolates with high biofilm levels do not necessarily outcompete UTI strains

Previous studies using well-characterized *E. coli* isolates 83792 [49,51,79] and Nissle 1917 [80] indicated that certain ASB or probiotic strains can potentially be used as prophylaxis against UTI. Given the striking diversity of biofilm formation in ASB-associated VUTI strains, we tested the ability of one particular isolate—VUTI 170, a strain which exhibited high surface-associated biofilm levels (Fig. 3b, **green dot**)—to persist in the bladder and outcompete the cystitis isolate UTI89 (Fig. 3b, **blue dot**). Although a domesticated laboratory strain, UTI89 is a classic strain used for investigating UPEC pathogenesis in animal models [81–83] and represents an average biofilm former based on the collected data from this study (Fig. 3b, **blue dot**). In addition to VUTI 170, four additional ASB VUTI strains with variable levels of biofilm formation were randomly selected for assessing colonization in the murine bladder. First, to evaluate colonization potential, the five different VUTI ASB isolates were inoculated transurethrally into 7–8 week-old female C3H/HeN mice, as previously described [81–83]. By 24 h postinoculation, VUTI 170 colonized the bladder at levels higher than the other ASB strains and at a comparable level to UTI89 (Fig. 5a). However, in subsequent coinfection experiments, UTI89 outcompeted VUTI 170 by as much as tenfold at 24 h postinfection (Fig. 5b), suggesting that the ability to form high levels of biofilm *in vitro* does not necessarily correlate with protecting the host from infection by a uropathogen. Collectively, these data strongly suggest that ASB isolates are as variable in their biofilm-forming ability as UPEC strains. Biofilm formation *in vitro* cannot be used as a simple indicator of virulence or probiotic potential. In general, the observed heterogeneity highlights the challenge of associating any one phenotypic trait with clinical symptomatology. Nevertheless, if more complex or nuanced strain-based criteria could be

established for differentiating UTI and ASB isolates, they would constitute a *de facto* molecular definition of UPEC itself.

Clinical isolates demonstrate metabolic signatures that distinguish ASB- from UTI-associated *E. coli* strains

In clinical practice, the identification and characterization of diagnostic microbial isolates must proceed not only accurately, but as rapidly as possible, to support patient care. Recent technologies that characterize isolates from their genetic or proteomic profiles represent a major technologic advancement [40,61,84]. Unfortunately, neither of these modalities nor defined phenotypic metrics are presently capable of differentiating ASB-associated strains of *E. coli* from symptom-causing UPEC (as discussed above). We hypothesized that metabolite usage may be a distinguishing factor, based on previous studies reporting specific UPEC requirements during *in vivo* infection using one or two well-characterized UPEC or ASB strains [52–54,81,83,85–87].

In collaboration with the Center for Innovative Technology (CIT) at Vanderbilt University, we designed an experiment to assess the metabolic profiles of the VUTI strains. For these experiments, we selected all ASB and cystitis isolates from cases with no other comorbidities as per the EHR (see also accompanying data-in-brief article). These amounted to 26 ASB and 77 cystitis isolates (Fig. 6a). To simulate the bladder environment, each strain was grown in pooled human urine and under hypoxic conditions [88]. When strains reached logarithmic growth phase (at ~7h), individual isolates were pooled together in groups of eight (Data in brief, data worksheet 4), sedimented to separate the supernatant and pellet fractions and subjected to a global metabolomics workflow using reverse-phase liquid chromatography tandem mass spectrometry (LC-MS/MS) (Fig. 6b).

The supernatant fractions were analyzed first, given that in clinical practice it is far more practical to evaluate urine for diagnosis. A total of 6711 compounds were detected in the analysis of urine extracts (extracellular milieu). Fig. 7a shows distinct clustering of the supernatant of the ASB samples and the cystitis samples in a principal component analysis (PCA). Filtering by a *p*-value <0.05 and a *q*-value of <0.1 narrowed the list to 582 significant compounds (Fig. 7b). Heat map visualizations also revealed differences in the metabolic profile of ASB isolates compared with cystitis isolates (Fig. 7b). Detected compounds were searched against a number of metabolite libraries and databases by ProGenesis 2.3 software (see also *Materials and Methods*) and were assigned candidate annotations and confidence levels, as described in Schrimpe-Rutledge et al. [89]. Data in brief worksheet 5 contains the top candidate annotations for all 429 significant compounds with a database hit. The global PCA and heat map visualizations revealed apparent differences in the metabolic profile of ASB isolates compared with cystitis strains (Fig. 7a–c). These observations were validated in a subsequent metabolomics analysis, in which ASB and cystitis isolate pools were scrambled for statistical robustness. Supplementary Table 1 summarizes the statistically significant compounds found in pairwise comparisons of ASB vs cystitis in the repooled samples.

Based on our previous studies that elucidated a dependence on *de novo* purine synthesis for intracellular UPEC survival [55], we focused on reproducible differences in purine

metabolism between ASB and cystitis isolates. Further interrogation of known intermediates in the purine metabolic pathway suggests that there is a difference in usage of the purine salvage and *de novo* pathways (Figs. 8 and S2) by cystitis isolates and revealed an interesting observation: Although cystitis isolates appear to deplete adenosine-related intermediates from the extracellular milieu (Fig. 8b), they do not uptake as much guanosine-related species from the media (Fig. 8c). Additional efforts focused on the interrogation of purine intermediates in the complementary pellet samples from ASB and cystitis isolates indicated no marked differences in the abundance of these metabolites (Fig. 8d–e), suggesting that the strains reach homeostasis, but possibly via different mechanisms that could be used to distinguish them diagnostically.

We previously reported that *de novo* purine synthesis is critical for intracellular expansion within the bladder niche [55]. In those studies, we evaluated the effects of *purF* and *purH* deletions, both of which were defective in their ability to establish intracellular bacterial communities in a murine model of infection [55]. Both of these enzymes reside upstream of the intermediates detected in the metabolomics analyses (Fig. 8a). The present data suggest that salvage of adenosine by UPEC strains may be sufficient to complement their purine needs (Fig. 8) via eventual conversion of adenosine to xanthosine monophosphate, GMP, and guanosine (Fig. 8a). Collectively, these metabolomics analyses point towards the potential use of a combination of purine biosynthesis metabolites—along with hydroxypropionyl carnitine and N-undecanoylglycine—as biomarkers to aid in predicting colonization by a cystitis versus an ASB strain.

Discussion

We are faced with a pressing need for rigorous metrics that can distinguish between ASB-associated bacterial strains and true, symptom-causing uropathogens, especially as rates of antibiotic resistance continue to rise. The current study highlights the power in harnessing the quantity and diversity of clinical isolates as wild-type resources, as we provide evidence for conserved metabolic differences between asymptomatic and symptom-causing urinary tract isolates. The organisms utilized here represent one of the largest collections to date of urinary *E. coli* isolates applied to basic investigation, and yet they still represent only a small fraction of such isolates—one month's worth—that are continuously generated by our medical center as part of routine patient care. Critically, the availability of deidentified patient data facilitates in-depth analyses that connect microbiologic traits and multiomic data to complex clinical phenotypes: in this case, asymptomatic *versus* symptomatic bacteriuria, but moving forward can also expand to other clinical scenarios such as diabetes, pregnancy, or ascending infection. In fact, building on this study, we have recently launched a programmatic initiative (the *Vanderbilt Urologic Infection Repository*) in which all diagnostic urinary tract isolates (*E. coli* and other) are being banked with deidentified linkage to underlying clinical parameters.

Previously, our work has demonstrated phenotypic heterogeneity among clinical isolates in their production of biofilm factors, including type 1 pili, cellulose, and curli [88]. However, no patient data were associated with those isolates [88]. Here, we are able to group isolates by patient symptom status, which allows us to look for predictors of disease state or identify

potentially diagnostic biomarkers. This study demonstrates that—although biofilm formation is central to the pathogenic cascade *in vivo*—utilizing *in vitro* biofilm abundance is *not* a predictor of disease state. Similarly, PCR-based probing of other bona fide virulence factor genes, such as the *pap* operon, also revealed no differences in gene carriage in cystitis versus ASB isolates (Fig. S3). From our extensive strain collection, we were also able to design commensurate metabolomics experiments, which revealed conserved differences in the metabolic profile of the supernatant fractions for ASB *versus* cystitis isolates (Figs. 6–8). Intriguingly, these studies show that guanosine levels are found at lower levels in the supernatant of ASB isolates, while adenosine levels are found at higher levels in the supernatant of ASB isolates. Guanosine and adenosine are part of purine metabolism pathway, and our lab has previously reported purine biosynthesis is necessary for UPEC intracellular survival [55]. Higher levels of the free purine base guanosine in the supernatant of cystitis isolates suggest that these strains are adapted for purine catabolism, facilitating intracellular survival in the bladder. Ongoing work is now dedicated to mining additional hits within these signatures, to identify and analyze additional metabolites in purine biosynthesis that could be used as distinguishing factors between UTI and ASB strains (Fig. S2).

Previous studies established the need for *guaBA* genes for growth of UPEC in human urine [86,87]. Moreover—and in support of our observations—work by Hagan et al. that assessed *in vivo* expression of UPEC genes in women with UTI-detected high expression of the *guaA* and *guaB* genes in the majority (but not all) of isolates tested [90]. Indeed, comparison of *guaA* transcript levels in a randomly selected cohort of ASB and cystitis isolates from our study, using quantitative PCR revealed consistent, low-level expression of *guaA* in the ASB isolates (Fig. S4). However, similar to the Hagan et al., study, steady-state transcripts were far more variable in the cystitis isolates, with three out of the five isolates tested expressing high levels of *guaA*, and two isolates expressing similar levels as the ASB strains (Fig. S4). These results highlight that qPCR-based differentiation of ASB and cystitis strains based on *guaA* expression alone would be unreliable and that careful examination of metabolite abundance or flux may prove more suitable for rapid identification of ASB and cystitis strains. Previous studies identified two inner membrane transporters responsible for nucleoside uptake: NupC and NupG [91]. Although NupG is able to transport all nucleosides, NupC does not import guanosine, raising the possibility that differences in NupC function or expression may be influencing differential uptake of nucleosides between ASB and cystitis strains [91–96]. We compared the NupG/C protein sequences and corresponding gene promoters in ASB and cystitis isolates in the metabolomics study that were also subjected to whole genome sequencing, but identified no differences at the genome level, consistent with the extensive variability in this species. As we obtain more genomic sequences, for all our collected isolates, it will also be intriguing to compare transcriptome differences as they may be directed by differential effects of master regulators. This is an area of current investigation in our groups.

From a mechanistic standpoint, our present study elucidates one more piece in the potential mechanism by which UPEC strains maintain nutrient balance during infection. Although in the bladder lumen, the salvaging of adenosine—which can be converted to inosine via the action of *add* (Fig. 8a and [87,91])—may be sufficient in allowing the cystitis isolates to

fulfill their purine needs, *de novo* synthesis is critical for intracellular expansion [55]. Investigation of the extracellular inosine inventory from the metabolomics studies indicated no differences in the abundance of inosine in the supernatant fractions from ASB and cystitis isolates. Although salvaging of adenosine appears to be sufficient for growth in urine, transition of UPEC strains into the intracellular niche necessitates the utilization of *de novo* synthesis [55], suggesting that free adenosines become scarce within the host cell niche.

Going forward, we will employ genomics to investigate differences in the genes associated with regulation of purine metabolism, in addition to those genes encoding purine metabolism enzymes in both ASB and cystitis strain backgrounds (Fig. 8a). Bridging pathogenesis and diagnosis, moreover, we will explore the ability of these metabolic signatures to serve as prognostic markers for evaluating the inherent pathogenicity of urinary *E. coli* isolates. For clinical cases where assessing symptomatology—or assigning symptoms to a urinary etiology—is challenging, empiric molecular criteria are sorely needed that can adjudicate a urinary strain as UTI- or ASB-like. The current work suggests that such criteria are achievable on a metabolomic level, although the diagnostic value of such an assay will rest on its performance characteristics at the level of individual strains. Building on the pools of strains employed here, future work will explore the phenomenon and its predictive value on a single-isolate level. In this regard, introducing small-molecule mass spectrometry (LC-MS) to the setting of clinical microbiology would represent a notable complement to the recent advent of protein mass spectrometry (MALDI-TOF) in diagnostic bacteriology for taxonomic identification [84,97].

Throughout this work, the ultimate strength lies in its repurposing of wild-type diagnostic isolates for patient-centered microbial discovery. The complexity and diversity of urinary *E. coli*—together with the clinical pictures associated with them—epitomize the need for systemic analyses that network the molecular profiles of human-associated microbes directly to the clinical phenotypes of the source patients. The sheer volume of urinary isolates that arise within major medical centers as laboratory derivatives of care creates a unique opportunity of leveraging these isolates for necessary translational studies. In total, therefore, we seek to overcome a complex clinical challenge by harnessing personalized microbial resources, expanding diagnostic and pathogenic understanding of *E. coli* in the urinary tract, and providing new resources for patients who suffer from UTIs.

Materials and Methods

Urine-associated *E. coli* collection

Urine-associated *E. coli* isolates and patient data were collected in accordance with Vanderbilt IRB #151465. Strains were initially isolated by the VUMC Clinical Microbiology Laboratory (a CLIA/CAP certified facility) via guideline-recommended semi-quantitative aerobic culture [58], as described above in the *Study Design* sub-section of the *Results*. These isolates represented the routine derivatives of patient care, with no urine specimens collected for the express purposes of this study.

Other bacterial strains

Model cystitis isolate UTI89 [98] was used in all phenotypic analyses as a positive control, given that its biofilm properties have been well-characterized *in vitro* and *in vivo* [60,99]. Additional characterized isolates used include CFT073 [100], EC958 [101], and ABU 83972 [79].

Biofilm assays

All strains were grown overnight in Lysogeny Broth (LB) (Fisher) pH 7.4 at 37 °C with shaking, unless otherwise specified. For liquid growth biofilms at the surface interface, total abundance (comprising the bacteria themselves and associated extracellular matrix) was quantified using the crystal violet method of O'Toole [102]. Overnight cultures were diluted to OD₆₀₀ = 0.05 and a volume of 100 µL was transferred to a 96 well PVC plate. Biofilms were rinsed in sterile water, stained with 0.5% crystal violet, and disaggregated with 35% acetic acid after 48 h. Absorbance of the disaggregated biofilm was measured at 570 nm using a SpectraMax i3 plate reader (Molecular Devices). Data represent an average of 3 biological replicates per isolate with at least 8 technical replicates. Statistical analysis was performed on GraphPad Prism using a Kruskal-Wallis one-way ANOVA. Colony biofilms were seeded using 10 µL of overnight culture that was spotted onto 1.2× YESCA CR agar and allowed to grow at room temperature and ambient atmosphere for a period of 11 days. Colony biofilms were imaged after 11 days of incubation. Images represent at least two biological replicates. Description of CR uptake and rugose morphology was qualitatively recorded as described previously [75,76].

Murine infections

Murine infections were performed as previously described [82,103]. Briefly, each strain was inoculated into 5 mL LB broth, grown shaking for 4 h at 37 °C, and then diluted 1:1000 into 10 mL fresh LB broth and grown statically for 24 h at 37 °C. After 24 h, the culture was diluted 1:1000 again into 10 mL fresh LB broth and grown statically for 24 h at 37 °C. Each bacterial culture was normalized to contain 10⁷ CFU bacteria in 50 µL phosphate-buffered saline (PBS). Each 7e8 week-old C3H/HeN female mouse was inoculated transurethrally with 50 µL of normalized culture. Mice were sacrificed at 6 or 24 h post infection. Bladders were removed and homogenized for total CFU enumeration. The VUMC Institutional Animal Care and Use Committee (IACUC) approved all animal studies (protocol number M1500017–01). All procedures on animals were performed in accordance with all recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the IACUC.

Combined human urine

Urine was collected from healthy human volunteers in accordance with approved protocols. A healthy volunteer is defined as an individual who is urologically asymptomatic, not menstruating, and who has not taken antibiotics in the last 90 days. An equal volume of male and female urine was combined from multiple volunteers to create a standard *ex vivo* growth media for all metabolomic experiments; it was passed through a 0.22 µm filter before use.

Growth conditions for metabolomics analysis

For each isolate, a single colony from an agar dish was inoculated in 5 mL LB and shaken overnight at 37 °C under ambient atmospheric conditions. Cultures were then diluted 1:1000 in the combined human urine and grown for 6 h to mid-log phase (37 °C, shaking), under 4% oxygen to emulate the bladder environment. After 7 h, OD₆₀₀ of each isolate was measured—and cultures were normalized by volume to yield equal number of organisms from each strain—before pooling into groups of isolates (Supplementary Data—worksheets 4–5). CFUs were enumerated for each pool to confirm bacterial density (~10⁹ total *E. coli* per pool). Each pool was then centrifuged to separate the cellular (pellet) and supernatant fraction. Pellets and supernatants were flash frozen and stored at –80 °C until for metabolomic analysis. An uninoculated urine sample was included for analyses.

LC-MS sample preparation, metabolite data processing and analysis, and global, untargeted UPLC-MS/MS analysis

Please refer to the corresponding Data in brief article for detailed methodology of the global metabolic analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used:

ASB	asymptomatic bacteriuria
CAUTI	catheter-associated urinary tract infection
CFU	colony-forming unit
CIT	Center for Innovative Technology
CR	Congo red dye
EHR	electronic health record
LC-MS/MS	liquid chromatography—tandem mass spectrometry
MALDI-TOF	matrix-assisted laser desorption ionization-time of flight
MDR	multidrug resistant
PCA	principal component analysis

rUTI	recurrent urinary tract infection
UPEC	uropathogenic <i>Escherichia coli</i>
UTI	urinary tract infection
VUMC	Vanderbilt University Medical Center
VUTI	Vanderbilt Urinary Tract Isolate

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Fig. 1. Clinical characteristics of bacterial colonization of the urinary tract.

(a) Image depicts the clinical spectrum of disease that can occur as a result of bacterial colonization of the urinary tract. (b) Pie chart depicts the distribution of bacterial species isolated in 2017 from urine samples at the Vanderbilt University Medical Center (VUMC), as recorded by the VUMC clinical laboratory. In parentheses are the numbers (*n*) of strains isolated per species.

Fig. 2. Clinical laboratory workflow for collection and binning of Vanderbilt urinary tract isolates.

Escherichia coli isolates ($n = 303$) were collected and stored by the clinical microbiology laboratory following protocols set forth by the *microVU* initiative. The Medical Director mined the electronic health records (EHR) of each source patient for clinical phenotypes. This resulted in 3 groups: unclear ($n = 18$), UTI-associated ($n = 199$), and ASB-associated ($n = 86$). Isolates with unclear information ($n = 18$) were excluded from downstream analyses. The remaining 285 UTI- and ASB-associated isolates were characterized further. The table indicates the breakdown of the 285 VUTI isolates per sex, as a function of age range.

Fig. 3. Extracellular biofilm formation does not correlate with disease state.

(a) Pie chart depicts the distribution of clinical diagnoses associated with the 285 VUTI isolates analyzed in this study. Blue arrow indicates the UTI-associated isolates (putative cystitis, pyelonephritis, and bacteremia). (b) Graph depicts liquid phase, surface-associated biofilm formation by VUTI isolates, as measured using the crystal violet assay of O'Toole [102]. Green arrow points to VUTI 170 (green dot). Blue arrow points to VUTI 89 (blue dot). Inset, image of crystal violet stained biofilms on PVC plates used for this assay. Orange line depicts the median. No statistically significant differences were detected between groups. Data points represent an average of 3 biological replicates each with at least 8 technical replicates. Statistical analysis was performed on GraphPad Prism using a Kruskal-Wallis one-way ANOVA. The accompanying data-in-brief article includes the clinical information

and raw datasets for the liquid phase, surface-associated biofilm formation in Data sheets 1 and 2, respectively.

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Fig. 4. Heterogeneity in VUTI colony biofilm phenotypes. Heterogeneity in VUTI biofilm phenotypes.

(a–c) Graphs depicting the number of ASB and UTI-associated isolates that exhibited rugosity (a), Congo red uptake (b), or phenotypic instability (c) when grown as colony biofilms on YESCA agar. No statistically significant differences were detected between groups. Statistical analysis was performed on GraphPad Prism using Fisher's exact test. See accompanying Data in brief article Data sheet 3 for a complete list of colony biofilm phenotypes in the VUTI isolates. (d) Representative images of colony biofilms spotted on Yeast Extract/Casamino Acids agar supplemented with Congo red dye. Each row depicts representative phenotypes from VUTI strains isolated from patients with distinct clinical diagnoses.

Fig. 5. Murine colonization variability in select ASB isolates.

(a) Graph depicts bacterial burden in the bladders of 7–8 week-old female C3H/HeN mice 24 h post transurethral inoculation with UTI89 or one of the five indicated ASB VUTI strains. Line depicts the geometric mean. Dotted line represents the limit of detection. (c). Graph depicts the competitive index of strains UTI89 and VUTI 170 during coinfection studies. Mice were coinfectd with equal CFUs of UTI89::HK and VUTI 170. Competitive indices were calculated by determining the ratio of UTI89 to VUTI 170 in the output pool and input pool and obtained at 6 and 24 h, respectively.

Fig. 6. Workflow for Metabolic analyses of uncomplicated cystitis and ASB.

(a) Graph depicts liquid-phase, surface-associated biofilm formation by uncomplicated cystitis and ASB VUTI isolates (as described in Fig. 3b). (b) Uncomplicated isolates from (a) were grown individually in conditions mimicking the bladder environment, pooled in groups of 8, and then spun down into supernatant and pellet fractions. These fractions were then subjected to a reverse-phase liquid chromatography tandem mass spectrometry (LC-MS/MS), comprising a global metabolomics workflow. The groupings for metabolomics analyses are detailed in the accompanying Data in brief article.

Fig. 7. ASB and cystitis strains exhibit unique metabolic profiles.

(a) PCA plots obtained from metabolomic analysis of pooled ASB and cystitis strains grown under hypoxic conditions in pooled human urine. (b) Heat map of all metabolites with significant differences between ASB and cystitis pools. ASB pools (A–C) are denoted with a black bar, cystitis pools (D–L) are denoted with a gray bar. (c) Heat map depicting all select purine synthesis and fatty acid metabolites. See also accompanying Data in brief article Data sheet 5.

(a) Image depicting the purine biosynthesis pathway (adapted from KEGG) in strain UTI89. In red are metabolites found to be elevated in the extracellular milieu of cystitis isolates relative to ASB isolates. In blue are metabolites found to be decreased in cystitis isolates relative to ASB isolates. (b–e) Graphs depict normalized concentration of adenosine (b, d) and guanosine (c, e) in the extracellular- (b, c) and cellular milieu (d, e). See also Table S1.