The iron hand of uropathogenic *Escherichia coli*: the role of transition metal control in virulence

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The role of iron as a critical nutrient in pathogenic bacteria is widely regarded as having driven selection for iron acquisition systems among uropathogenic *Escherichia coli* (UPEC) isolates. Carriage of multiple transition metal acquisition systems in UPEC suggests that the human urinary tract manipulates metal-ion availability in many ways to resist infection. For siderophore systems in particular, recent studies have identified new roles for siderophore copper binding as well as production of siderophore-like inhibitors of iron uptake by other, competing bacterial species. Among these is a process of nutritional passivation of metal ions, in which uropathogens access these vital nutrients while simultaneously protecting themselves from their toxic potential. Here, we review these new findings within the current understanding of UPEC transition metal acquisition.

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Bacterial metal acquisition has long been understood to play an important role in infection pathogenesis. Historically, most studies in this area have focused on host–pathogen competition for iron, a critical nutrient for most microbes. Recent studies have begun to consider how the full range of biologically relevant transition metals is handled at the host–microbe interface. In this review, we will consider host–bacteria metal interactions as they relate to human

urinary tract infections (UTIs). We will focus primarily on lessons from *Escherichia coli*, the most common cause of complicated and uncomplicated UTIs. First, we will review recent findings on how human hosts manipulate metal availability during infection. Next, we will cover the many strategies used by uropathogenic *E. coli* (UPEC) to control transition metal ions during colonization and infection. Prominent among these bacterial strategies have been small molecule chelators produced by the bacteria – siderophores and metallophores – with virulence-associated activities. While much remains unknown, a picture of myriad metal interactions in uropathogenesis extending well beyond iron alone is coming into focus.

Uropathogenic *Escherichia coli*

Urinary tract infections

UTIs are among the most commonly encountered infections in both outpatient and inpatient settings. While females are at greater lifetime risk for UTI, males exhibit a delayed, age-related increase in incidence. Oral antibiotics are the mainstay of UTI treatment and, in females without risk factors ('uncomplicated UTI'), antibiotics are given for the purpose of minimizing symptoms. Urinary tract abnormalities, catheterization and diabetes (a few of the factors leading to designation as 'complicated UTI') increase both UTI risk and risk of progression to more severe infection. A minority of UTIs will progress to pyelonephritis (kidney infection) and even septicemia, sometimes due to anatomic abnormalities but often for unclear reasons. The dramatic expansion of antibiotic-resistant uropathogens is complicating UTI management, driving interest in new diagnostic and therapeutic approaches. Although a variety of uropathogens can cause UTI, *E. coli* is the most commonly isolated pathogen, with related *Enterobacteriaceae* such as *Klebsiella*, *Enterobacter* and *Citrobacter* comprising many of the remaining cases, particularly in hospital settings. *Escherichia coli* is thus the most widely studied uropathogen [1].

Escherichia coli UTI pathogenesis

The ascending infection model developed in the late 1970s by Thomas Stamey remains the dominant pathophysiological paradigm for UTI pathophysiology [2]. In females, the normal intestinal reservoir of colonizing *E. coli* first asymptotically colonizes the vaginal mucosa. Next, urethral ascent to the bladder lumen results in detectable urinary bacteria (bacteriuria). This stage may either be asymptomatic, and not clinically regarded as an infection (asymptomatic bacteriuria), or symptomatic, meeting the clinical definition of bladder infection (cystitis). In a subset of these patients, *E. coli* ascends further to reach the kidneys, resulting in kidney infection (pyelonephritis), a more severe infection with potential to extend to the blood stream (septicemia). The ascending infection model thus requires infecting *E. coli* to successfully occupy and traverse a wide variety of host environments. In the intestine, oxygen can be low and *E. coli* generally exists with a diverse microbiome. Upon colonization of the vaginal mucosa, *E. coli* encounters a different microbiome, a different mucosal surface, and variable oxygenation levels. The luminal environment of the urinary tract consists of urine and a urine-exposed mucosal layer overlaying more specialized epithelial cell populations. The urinary component of these environments exhibits substantial compositional variation of ions, metabolites, proteins and cells. With tissue invasion, *E. coli* reaches interstitial spaces and even intracellular sites, where they occupy lysosome-like vesicles [3] or form biofilm-like intracellular bacterial communities (IBCs) in epithelial cells [4]. Resident and recruited phagocytic cells, such as neutrophils and macrophages, create additional specialized microenvironments, such as phagolysosomes, likely to be relevant to UTI. While these environments are likely to exert substantial selective pressures on *E. coli*, we continue to learn more about their relevant features.

Virulence factors of UPEC

E. coli are genetically diverse, and urinary isolates tend to possess distinctive genetic features linked to virulence. Nearly 30% of open reading frames in *E. coli* UTI89, a model UPEC strain isolated from a patient with acute cystitis, are not found in strain MG1655, a derivative of the original K12 strain that was obtained from a fecal specimen in 1922 [5,6]. Urinary *E. coli* isolates from UTI patients similarly retain substantial genetic diversity, and their designation as UPEC is currently better regarded as a functional, rather than genetic, classification. Studies have linked both nonconserved genes (variable genes) and polymorphisms in conserved genes (core genes) to UPEC isolates. In studies of UPEC, nonconserved genes with putative disease-associated functions are generally designated as virulence factors (VFs), although microbiologists often use this term more broadly to describe any bacterial function that facilitates infection [7]. We will use the former definition in this review. VFs encompass a diverse range of functions ranging from enhancement of motility and adhesion, to toxin secretion and immune evasion,

to nutrient acquisition (for review see [8]). Reliable monogenic VF associations with UTI are not observed, which may reflect individualistic selective pressures in each host, inaccuracies in clinically distinguishing asymptomatic bacteriuria from UTI, or a requirement for some VFs to exist within a larger synergistic group of VFs to manifest a phenotype. Indeed, when contemporary network approaches were used to discern VF combinations in 337 inpatient urinary *E. coli* isolates, stereotypical VF repertoires (mathematically termed as ‘communities’) were identified [9]. These communities were associated with patient sex and antibiotic resistance. One interpretation of these observations is that evolutionary pressures favor synergistic VF combinations, rather than individual VFs, that act as distinctive pathogenic ‘strategies’ during *E. coli* uropathogenesis. Interestingly, each VF community possessed a different siderophore system which indicated the presence of the other, co-associated VFs.

Additional evidence connecting transition metal interactions to clinical *E. coli* pathogenesis comes from transcriptional analysis of voided bacteria recovered from cystitis patients. These studies detected upregulation of multiple iron acquisition systems and a nickel uptake system in patients [10,11]. Transcriptional analysis of UTI89 within IBCs from a murine cystitis model further revealed that siderophore system genes are among the highest upregulated genes [12]. While a wide variety of bacterial factors are undoubtedly necessary for urinary virulence, multiple lines of genetic data support a closer look at the role of transition metal interactions in UPEC as a means to better understand how UPEC defeats the physiological barriers to UTI pathogenesis.

Bacterial demands for metals during infection

Metal ions are critical co-factors for around 40% of enzymes with known structures. Analysis of a representative subset of well-studied metalloenzymes suggests that metal cofactors directly catalyze redox chemistry for approximately a third of metalloenzymes [13]. Metals not involved with redox catalysis can still serve to activate a reactant, stabilize an intermediate or contribute to the overall protein structure [14]. Nutritional requirements for metal ions may change with the bacterial environment. Iron, in particular, is a classic growth-limiting nutrient of most free-living bacteria. Nickel is a critical cofactor for multiple anaerobic metabolism enzymes [15]. Adoption of aerobic respiration by facultative anaerobes, such as *E. coli* during movement from the gut to the urinary tract, will likely result in increased metal demands [16]. Redox enzymes are necessary both for electron transport chains during aerobiosis and for detoxification of reactive oxygen species. Biosynthesis of these metalloenzymes may be particularly consequential when UPEC encounters reactive oxygen species generated through the respiratory burst of phagocytic cells during active infections [17]. Transition metals such as Fe(III), Mn(II) and Cu(II) are necessary for the activity of superoxide dismutases (SODs), reductases, peroxidases and catalases [18]. In fact, manganese has been observed to help *E. coli* tolerate oxidative stress [19]. Manganese uptake is activated by H₂O₂ and it appears to protect against oxidative damage by activating Mn-SOD and metallating damaged iron-dependent enzymes [20]. The prominent role of metalloenzymes in tolerating infection-associated microenvironments suggests a particularly consequential role for microbial metal acquisition during infection pathogenesis.

Metal ion sources in the host

Host metal homeostasis

A substantial body of literature exists regarding iron homeostasis in humans, and this remains an active area of investigation. In reducing conditions such as anoxic environments, iron exists predominantly as reduced ferrous (Fe(II)) ions, which are relatively soluble. However, in oxic environments iron is oxidized, resulting in poorly soluble ferric (Fe(III)) ions, which form complexes of varying stability with most biomolecules. In human hosts, iron exists predominantly as ions complexed with heme or proteins, or in nonspecific complexes. The majority of heme molecules exist within intracellular protein complexes such as hemoglobin, myoglobin and cytochromes. The toxicity associated with free iron ions is largely controlled by the host through sequestration in proteins. A substantial fraction of nonheme iron is bound to intracellular ferritin, a 480 kDa protein which can bind up to 4500 Fe(III) ions [21]. Ferritin-bound iron is mobilized to the extracellular iron-binding protein transferrin, which can bind two ferric ions to form the predominant circulating form of iron. The degree of transferrin iron saturation is a commonly used metric of clinical iron status. The iron content of human urine is normally very low (~0.1 μmol per liter) in the uninfected state [22]. Even in the absence of inflammatory stimuli, iron is subject to tight physiologic control.

Copper, zinc and nickel homeostasis are less well understood and the subject of active research. As with iron, these metals each have toxic potential and their free concentrations are subject to tight physiologic control. Ceruloplasmin is a cuproenzyme and the major form of circulating copper, accounting for >95% of plasma copper content [23].

Copper enters mammalian cells through members of the Ctr family of copper transporters and is subsequently delivered to cuproproteins via chaperones (Cox17, CCS, Atox1). ATP7A/B are P-type ATPases which serve to transport copper into vesicles or out of the cell, as needed [24]. Zinc is imported into mammalian cells via ZIP proteins of the SLC39 family [25] and is exported via Znt transport proteins of the SLC30 family. Znt proteins also facilitate loading of zinc into intracellular vesicles for utilization or storage [26,27]. Cytosolic copper and zinc can be bound by metallothioneins – a diverse class of proteins that may be involved in preventing metal toxicity, although their physiological functions are poorly understood and thought to be diverse [28]. The total transition metal content of human urine (per liter), is low, with urinary zinc content (~ 4 μ moles) exceeding that of copper (~ 0.2 μ moles), nickel (~ 0.04 μ moles) and manganese (~ 6 nmoles) [29]. Total urinary copper content increases during human and primate UTI, with ceruloplasmin accounting for a substantial portion of that increase [30,31].

Nutritional immunity

Nutritional immunity is a biological strategy in which the host restricts nutrient availability to microbes. Iron sequestration, a prototypical nutritional immune strategy, is accentuated in the host during infection. Systemic inflammation is associated with elevated circulating levels of hepcidin, a peptide hormone that induces a drop in serum iron content through intracellular sequestration and reduced dietary absorption [32]. Hepcidin knockout mice display symptoms of iron overload (hemochromatosis) and increased susceptibility to systemic infection by the siderophore-producing Gram-negative pathogen *Vibrio vulnificus* [33]. Metal ion transport can also act locally to control bacterial pathogens. The integral membrane protein NRAMP1 is localized to the phagolysosomal membrane, where it removes transition metals (iron and manganese) from the phagosome, denying these nutrients to engulfed bacteria [34]. Metal transport in these contexts is one important component of nutritional immunity.

Inflammation brings numerous metal-binding proteins to the infected urinary tract. We define these metal-binding proteins as proteins with metal binding capacity that are secreted in their metal-free state in order to bind bioavailable metals at the site of inflammation, sequestering them from pathogens. Lactoferrin is a member of the transferrin family (61% sequence identity with transferrin) that is secreted by activated neutrophils and observed in urine from UTI patients [35]. Lactoferrin binds its two iron ions 300-times more tightly than transferrin [36]. The metal-binding protein complex calprotectin (a S100A8/S100A9 heterodimer) has also been associated with UTIs [37]. Initially appreciated to bind zinc and manganese [38], calprotectin has since been found to bind numerous transition metals, blocking bacterial access. A recent study found that extracellular Ca(II) allosterically stimulates binding of ferrous iron to calprotectin at a distinct site. This is unusual in that most iron sequestration proteins are known to sequester oxidized, ferric iron [39]. A similar activity was observed for Ni(II), starving *Staphylococcus aureus* of this metal [40]. Lactoferrin and calprotectin are thus examples of host proteins that may starve uropathogens of transition metals through direct metal ion binding.

Also increased in urine from UTI patients is siderocalin (SCN; also known as lipocalin-2/LCN2, neutrophil gelatinase-associated lipocalin/NGAL, or 24p3) [22,41], a soluble protein released by neutrophils and secreted by urinary epithelial cells in response to inflammatory stimuli [42,43]. SCN indirectly binds ferric ions through various small molecule chelators, motivating its renaming as SCN [44]. Urinary SCN levels are significantly higher in *E. coli* UTI patients than in asymptomatic controls [22,41]. In environments approximating human serum conditions, SCN can bind and sequester ferric complexes with enterobactin, the conserved *E. coli* siderophore [44,45]. However, in human urine with pH >6.5, SCN instead binds ferric ions through urinary catechol metabolites, forming a stable ferric–catechol complex. The ability of SCN to starve urinary *E. coli* of iron accordingly varies widely with the urinary catechol content [22,46]. The antibacterial mechanism of SCN may thus vary during infection or between individuals, depending upon urinary composition or tissue localization.

UPEC metal acquisition systems

Competition for metal ions between host and pathogens appears to have driven substantial evolutionary adaptation by both *E. coli* and mammalian hosts. Metal scarcity is recognized in *E. coli* through conserved genetic systems, of which Fur (ferric uptake regulator) is best understood. Analogous transcriptional regulators, Zur, MntR and NikR, regulate zinc, manganese and nickel uptake, respectively [47–49]. These systems transcriptionally activate a variety of metal uptake genes in *E. coli*, can be functionally redundant, and are still being discovered. In this review, we classify metal import systems into three groups (Figure 1): import of uncomplexed free metal ions; import of metal ion complexes found in the host; and import of metal ions complexed with *E. coli*-produced chelators called

Figure 1. Uropathogenic *Escherichia coli* metal acquisition strategies. Three strategies of metal import are illustrated, with associated systems and transport substrates indicated below. Each system requires porins or dedicated outer membrane receptors as well as dedicated inner membrane transporters (cylinders) to transport either the bare metal (red sphere) or metal complexes (gray and blue complexes).

siderophores. We will focus on those systems which have been directly tied to UPEC virulence, although novel putative metal acquisition systems are still being discovered in UPEC [50–52].

Import of free metal ions

If a metal ion is sufficiently soluble and abundant in a host infection environment, UPEC will utilize transporters dedicated to import of free (uncomplexed) ions. A number of these transporters have been associated with UTI pathogenesis. Metals transported in this way include ferrous iron, manganese, zinc and nickel. A number of metal ion transporters are capable of transporting multiple ions *in vitro*, which may obscure their physiological substrates or indicate a degree of functional redundancy. The majority of studies in this field have focused on the inner membrane transporters with the assumption that the metal ions are permeable to the outer membrane.

Reduced ferrous iron (Fe(II)) is more abundant in anoxic environments and is more soluble than oxidized ferric iron. Thus, in environments where ferrous iron is abundant, its direct import is plausible. At least five transporters in *E. coli*, FeoABC, SitABCD, MntH, EfeUOB and ZupT are capable of importing ferrous iron [53–58]. Of these, the Sit system specifically has been tied to UPEC virulence. Sit is present in two widely used UPEC model strains (UTI89 and CFT073), and its expression is enhanced during experimental murine cystitis [43,53]. Both SitABCD and MntH are also capable of manganese import [57,59,60], although their physiological substrates during UTI are unclear.

Nonferric divalent metal ion importers have also been implicated in animal models of *E. coli* UTI. The high affinity zinc importer, ZnuABC is important for *in vitro* UPEC growth in zinc-deficient conditions [61] and deletion mutants lacking *znuABC* exhibit diminished virulence [62]. The nickel import system, Nik, is upregulated in *E. coli* voided from female cystitis patients, and UPEC mutants lacking this system also exhibit a virulence defect [11].

Import of metal ion complexes in the host

Some metal ions interact with small molecules and form complexes that are soluble in the host, making these complexes nutritionally valuable to UPEC. UPEC systems that import these complexes stereotypically include an

outer membrane receptor which imports the molecules into the periplasm using energy from the proton gradient across the inner membrane by coupling to the inner membrane TonB/ExbB/ExbD complex. Once in the periplasm, specific inner membrane transporters facilitate import or metal acquisition from these complexes.

Heme is the most abundant iron complex in the host, and heme receptors accordingly have a well-substantiated connection to UPEC. The Chu and Hma systems both import heme and are more prevalent in UPEC compared with commensal strains [63–66]. Transcriptional analysis of UPEC isolated from human infection reveals that *chuA* is highly upregulated, while *hma* was not as consistently or as highly upregulated [67]. *chuA* has been shown to be upregulated in murine UTI models [43,53]. An *hma* CFT073 mutant has decreased virulence in the kidneys in a murine co-infection model, and deletion of both receptors decreases kidney colonization [66]. Heme importers thus appear to play an important role in UPEC pathogenesis.

Other such UPEC systems with potential relevance to UTI are the Fec and Fhu systems. Fec imports ferric-citrate complexes, potentially taking advantage of the natural abundance of citrate in human urine and plasma, and its affinity for ferric ions [68–70]. The relevant substrate for Fhu during infection is less clear. Fhu genes (*fhuABCDE*) are capable of importing an array of ferric complexes, including the bacterial siderophore aerobactin and the fungal siderophores ferrichrome, coprogen, and rhodotorulic acid [71–73]. The *fhuA* gene emerged from an unbiased search for positively selected genes in UPEC [74], and *fhuA* and *fhuC* upregulation was observed in a murine UTI model [53]. Both FhuA and FhuE were detected on the cell surface of 44 and 11% of clinical UPEC isolates, respectively [50]. Whether the Fec and Fhu systems confer provirulence activities to UPEC remains unclear.

Siderophores

In addition to importing host metal complexes, UPEC also creates new metal complexes by synthesizing distinctive high-affinity small molecule metal chelators with higher metal ion affinities than many host proteins [75]. Bacterial chelators that bind ferric ions were among the first to be recognized. These were called siderophores which is Greek for ‘iron carrier’. Siderophores share an extraordinarily high affinity for ferric ions yet are chemically diverse. Following secretion by bacteria, they bind Fe(III) and the resulting complex is imported through dedicated import systems very similar to those described above for host metal ion complexes.

Enterobactin, the conserved E. coli siderophore

Enterobactin (formerly known as enterochelin) is a prototypical siderophore produced by all intestinal *E. coli* isolates studied to date [76]. Its biosynthesis and uptake genes are regarded as part of the core *E. coli* genome. Nevertheless, enterobactin production is not unique to *E. coli* and has also been described among urinary isolates of *Klebsiella* [77], *Enterobacter* and *Citrobacter* [78]. An even wider variety of bacteria, some of which are recovered from the urinary tract, are capable of using ferric enterobactin as an iron source despite their inability to synthesize this siderophore, a concept sometimes termed siderophore ‘piracy’ [79]. Enterobactin chelates ferric iron using three covalently linked 2,3-dihydroxybenzoylserine (DHBS) catechol moieties, affording an extremely high affinity for iron (K_d of 10^{-52} M) (Figure 2) [80].

Multiple lines of inquiry implicate the enterobactin system as a participant in human UTI pathogenesis. Enterobactin has been directly detected in urine from *E. coli* UTI patients [46], and mRNA for the outer membrane ferric enterobactin importer (*fepA*) was found to be upregulated in voided *E. coli* from UTI patients [10]. Two outer membrane receptors (Fiu and CirA) for the enterobactin subunits 2,3-dihydroxybenzoate and DHBS [81] (Figure 2) have also been observed to have increased transcription during human UTI, though their role in pathogenesis remains unclear [10]. A comparative genomic analysis of conserved genes in clinical *E. coli* isolates yielded evidence of positive selection in genes encoding components of both enterobactin biosynthesis (*entD*, *entF*) and uptake (*fepE*) among urinary strains [74]. Together these findings indicate that the enterobactin system is active during human UTI and influences *E. coli* fitness sufficiently to have influenced evolution of urinary strains.

The exceptional ferric ion affinity of enterobactin makes it capable of successfully competing with ferric ions bound to mammalian iron binding proteins that appear in the human urinary tract. In serum-like environments SCN can sequester ferric enterobactin using a bacterium’s own siderophore to deprive it of iron [44,45]. In human urine, however, the SCN calyx instead uses urinary catechols to sequester iron. These catechols are host-derived urinary metabolites that exhibit marked individual differences in concentration. Enterobactin appears to be particularly capable of retrieving iron from these catechol–SCN complexes [22,46]. Because urinary conditions necessary for SCN to sequester iron–catechol complexes (pH >6.5 and the presence of specific unconjugated catechols) vary between human hosts, the virulence-associated gains of function conferred by the enterobactin system may

Figure 2. Uropathogenic *Escherichia coli* siderophore systems. The synthetic and utilization genes for each of the four uropathogenic *Escherichia coli* siderophore systems are indicated along with the type and structure of each siderophore. Chemical moieties involved in metal chelation are highlighted in orange. Distinctive chemical features are highlighted with colored circles. DHBS: 2,3-dihydroxybenzoylserine.

be strongly host dependent. Indeed, Paragas *et al.* have described a urinary acidification response (pH <6.0) to UTI in mice that would be expected to interfere with catechol-mediated, but perhaps not enterobactin-mediated iron sequestration by SCN [41]. The literature thus supports scenarios in which the chemical composition of UTI microenvironments determines whether enterobactin facilitates bacterial access to iron (when SCN uses catechols to bind iron) or iron deprivation (when SCN sequesters ferric enterobactin).

Salmochelin, a virulence-associated enterobactin derivative

Salmochelin is the most recently identified UPEC siderophore and is a modified form of enterobactin generated by a distinctive C-glucosylation of the catechols and hydrolysis of the enterobactin cyclic trilactone scaffold (Figure 2) [82,83]. Originally identified from *Salmonella* species, it is also produced by UPEC carrying the nonconserved *iroA* gene cluster in the genome or plasmids [84]. This gene cluster does not encode the genes necessary for total synthesis of salmochelin, instead, its biosynthetic genes encode an enzyme that couples glucose to enterobactin through an unusual carbon linkage (*iroB*) [82] and a periplasmic esterase (*iroE*) that linearizes cyclic versions of enterobactin and salmochelin prior to secretion [85,86]. The *iroA* cluster also encodes a distinct exporter, receptor and cytoplasmic esterase (Figure 2) necessary for salmochelin-mediated iron uptake [82,85–87].

Evidence of salmochelin expression during clinical UTI is limited to transcriptional detection of the mRNA encoding the outer membrane salmochelin importer (*iroN*) in the voided *E. coli* recovered from some female UTI patients [67]. Transcriptional profiling of IBCs isolated by laser microdissection in a murine UTI model detected a 45–234-fold increase in *iroA* gene transcription compared with the same bacterial strain recovered from the intestine [43]. When concurrent urinary and rectal isolates from uncomplicated female outpatients were compared, urinary isolates produced more salmochelin in a standard culture condition than their corresponding rectal strains [76]. In another study of males with febrile UTI, *E. coli* carrying the salmochelin import gene *iroN* were more common among urinary than concurrent rectal isolates [88]. Metabolomic profiling suggests that the cost of producing salmochelin may be much higher than the cost of producing enterobactin or Ybt. The persistence of salmochelin production among clinical *E. coli* isolates suggests that the adaptive benefit of this system may exceed its metabolic cost in some environments [89].

Converting enterobactin to salmochelin generates a larger and more hydrophilic siderophore [82]. This may minimize membrane partitioning and binding to hydrophobic surfaces, increasing salmochelin's value to UPEC in the host [90,91]. The addition of two bulky C-linked glucoses to enterobactin, in particular, sterically prevents its sequestration by SCN, a virulence-associated property observed in culture and in a murine infection model. In these models, the *iroA* gene cluster rescues SCN-mediated growth restriction [92]. Beyond its canonical siderophore function, the salmochelin outer membrane receptor, IroN, has specifically been linked to biofilm formation and

invasion of urothelial cells by UPEC through unclear mechanisms [93,94]. In the animal models examined to date, modest defects for *iroN*-null UPEC strains have been observed [95,96]. It remains unclear exactly where and how salmochelin is most influential during UTI pathogenesis.

Aerobactin, a citrate-derived siderophore

Aerobactin is a hydroxamate-type siderophore originally isolated from *Aerobacter aerogenes* that is structurally distinct from enterobactin, salmochelin and Ybt (Figure 2) [97]. The operon containing synthetic (*iucABCD*) and outer membrane receptor (*iutA*) genes is present in the chromosome or virulence-associated plasmids of UPEC [7,98], as well as a wide variety of other medical and environmental bacterial species. Aerobactin-mediated iron uptake involves separate, conserved *E. coli* genes (*fhuBCD*) implicated in uptake of ferric hydroxamates [73]. Unlike enterobactin and salmochelin, it appears that the metal can be removed from aerobactin in a nondestructive manner and that the cell recycles the metal-free aerobactin [73]. Although aerobactin has a lower affinity for iron than enterobactin [99], its association with virulence raises the possibility that it confers important gains of function during infections.

Evidence of aerobactin expression during clinical UTIs is suggested by transcriptional detection of RNA for the associated outer membrane aerobactin importer (*iutA*) in some voided *E. coli* recovered from female UTI patients [67]. However, because some *iutA*⁺ isolates do not synthesize aerobactin, the transcriptional data are not definitive evidence of aerobactin system expression during UTI [76]. Epidemiological studies indicate that aerobactin gene carriage is more frequent in isolates from the bladder, kidney and blood, than in fecal isolates [100]. Other epidemiologic studies have observed that aerobactin prevalence is higher in UPEC strains which cause same-strain recurrent infections [101,102]. A recent large study of inpatient urinary isolates found that *iucD* was associated with both fluoroquinolone resistance and male patients [9]. Genes encoding the aerobactin system have been noted in several UTI-associated multidrug-resistant (including fluoroquinolone resistance) *E. coli* strains, including *E. coli* ST131 [103]. The nature of association between the aerobactin system and antibiotic resistance remains unclear.

Specific gains-of-function that may be conferred by aerobactin over other UPEC siderophores are incompletely understood. In mouse UTI competition assays, *E. coli* mutants deficient in aerobactin uptake (*iutA*-deficient CFT073) compete poorly in the bladder and kidney with isogenic wild-type strains with intact enterobactin and salmochelin systems [104,105]. Aerobactin is relatively uninhibited in the presence of serum, perhaps due to a lower hydrophobicity and lack of aromatic character relative to enterobactin [73]. One study indicates a superior ability to steal iron from transferrin in the presence of serum [106]. Like salmochelin, aerobactin is resistant to SCN sequestration, another potentially virulence-associated property [45]. Unlike salmochelin, ferric ion binding to aerobactin is relatively insensitive to low pH within the physiologic range [107], perhaps compensating for its lower affinity at neutral pH.

Yersiniabactin, an enterobacterial siderophore & metallophore

Yersiniabactin (Ybt), as the name suggests, was originally identified in *Yersinia pestis* (the agent of black plague). Ybt system genes are encoded in a complex, multioperon pathogenicity island known as the *Yersinia* high pathogenicity island (HPI) which has been identified in multiple urinary *Enterobacteriaceae*, including *Klebsiella*, *Enterobacter* and *Citrobacter* [108]. Expression of the HPI's four operons is dependent upon YbtA, an HPI-encoded transcription factor [109], with an additional level of transcriptional control by the ferric uptake repressor. Ybt is structurally distinct from enterobactin, salmochelin and aerobactin. An extensive nonribosomal peptide synthase/polyketide synthase system assembles salicylic acid, three cysteines and malonic acid to generate the polycyclic Ybt and the recently appreciated product escherichelin (Figure 2) [110,111]. Careful studies of the Ybt uptake pathway indicate that UPEC can completely recycle Ybt, nondestructively separating Ybt from its metal cargo and returning the liberated Ybt to the extracellular space, where it may undergo additional rounds of metal delivery [112].

Yersinia HPI activity in *E. coli* has been directly linked to human UTI pathogenesis. Both Ybt and escherichelin have been unambiguously detected using mass spectrometry in urine from *E. coli* UTI patients [111,113]. Transcriptional profiling of voided *E. coli* suggests that the outer membrane ferric Ybt importer (*fyuA*) is expressed during clinical UTI [67]. In IBCs isolated by laser microdissection in a murine UTI model, the first biosynthetic enzyme in Ybt biosynthesis (*ybtS*) was the most highly upregulated gene observed (~2000-fold increase) when compared with expression in the same bacterial strain recovered from the intestine [43]. When concurrent urinary and rectal isolates from uncomplicated female outpatients were compared, more urinary isolates produced Ybt in a standard culture condition than concurrent rectal strains [76]. In another study of males with febrile UTI, *E. coli* carrying the

Ybt import gene *fyuA* were more common among urinary than concurrent rectal isolates [88]. Interestingly, both biofilm production and the *Yersinia* HPI were found to be independently associated with UPEC strains which caused same-strain recurrent UTI [101]. Together, these data support an active role for the *Yersinia* HPI in clinical UTIs.

While a role for Ybt in the virulence of *Yersinia pestis*, where it appears to be the sole siderophore system, is well documented [108], evidence for its virulence-associated activity in UTI is more recent. A UPEC strain lacking the outer membrane importer FyuA was found to exhibit a virulence defect in an acute mouse UTI model (48 h infection of CBA/J mice) [10]. In a mouse cystitis model with a bimodal infection outcome (high titer vs low titer bladder infection after 1+ week infection of C3H/HEN or C3H/HeOuJ mice), a uropathogen lacking the *Yersinia* HPI-encoded inner membrane transporters, YbtP and YbtQ, exhibited a remarkable, 5–6 log-fold CFU defect relative to isogenic wild type in the mice that proceeded to high titer infections [114]. Interestingly, no defect was resolved in low titer infections where inflammation was low. This is a remarkable defect for nonessential, nonconserved genes and suggests that YbtP and YbtQ play important roles in virulence, despite the presence of intact enterobactin and salmochelin systems. In its canonical iron-scavenging role, Ybt's resistance to sequestration by SCN is one possible explanation for this activity [115], although Ybt appears to be ineffective at removing iron from SCN/catechol complexes [22]. These data suggest a consequential role for metal-Ybt import proteins during UTI.

Noncanonical functions of the UPEC *Yersinia* HPI

Yersiniabactin is a metallophore

Recent data point to additional roles for Ybt beyond siderophore-mediated iron acquisition that may explain why UPEC often carry the *Yersinia* HPI alongside other siderophore systems. Ybt was found to readily form stable complexes with copper during both human and experimental murine UTI [113]. *In vitro* biometal screens found that Ybt forms stable complexes with iron, copper, cobalt and nickel *in vitro* [116]. The *Yersinia* HPI-encoded outer membrane receptor, FyuA, recognized each of these non-iron Ybt complexes and could import them into *E. coli* [116]. Subsequent work has shown that Cu(II)-Ybt import supports the activity of *E. coli* amine oxidase (TynA), a classic copper-dependent enzyme, demonstrating that Cu(II)-Ybt can serve as a nutrient source for *E. coli* [112]. This nutritional role was dependent upon expression of the inner membrane ABC transporters, YbtP and YbtQ. The *Yersinia* HPI is thus a urovirulence-associated exception to the oft-stated premise that *E. coli* lacks a copper import system. Although a stable Zn(II) complex has not been observed [116], indirect data involving the *Yersinia* HPI-encoded inner membrane permease YbtX suggest a role in zinc import through an as yet unidentified mechanism [117,118]. Together these studies provide compelling evidence that Ybt should be regarded not only as a siderophore but as a metallophore, capable of facilitating import of multiple transition metal ions for nutritional purposes. As such, Ybt appears to not be entirely functionally redundant with the other UPEC siderophores, as it originally appeared.

Metal toxicity & nutritional passivation by yersiniabactin

Copper is a distinctive biological metal recognized as both a bacterial nutrient and toxin. While copper is a key cofactor for multiple *E. coli* enzymes, it can also generate reactive oxygen species and, as reduced Cu(I) ions, damage cytoplasmic iron–sulfur clusters [119]. Indeed, *E. coli* has long been appreciated to possess multiple genetic systems that protect from copper toxicity by exporting cytoplasmic copper ions [120]. Detection of Cu(II)–Ybt complexes in the urine from cystitis patients initially led to an investigation of the role of Ybt in the context of copper toxicity. Ybt was found to protect UPEC from copper toxicity by binding to the copper ions and minimizing their reactivity – a concept subsequently termed ‘passivation’ in a biological extension of the term from chemistry, which denotes a chemical interaction leading to decreased reactivity [112,121]. Urinary *E. coli* isolates were found to be more copper resistant than contemporaneous rectal isolates in UTI patients [113]. In voided *E. coli* from UTI patients, the copper efflux system, CusCFBA and CusRS, was observed to be upregulated [11], consistent with active copper stress during UTI. Taken together, these observations suggest that Ybt secretion protects UPEC from copper-mediated stress during UTI. These findings suggested a role for copper toxicity in human UTI.

Precisely where, and how, copper passivation by Ybt might influence UPEC pathogenesis remains unclear. Of note, copper supplementation and deprivation of mice have recently been shown to decrease and increase, respectively, bacterial colonization of the bladder [11,30]. These observations may relate to general changes in labile or ‘free’ copper in the proximity of infecting bacteria. Trafficking of the copper ion transporter ATP7A

Figure 3. Nutritional passivation by a metallophore. A Gaussian curve (dark blue) represents bacterial fitness over a range of copper ion concentrations. Yersiniabactin–metal binding widens the curve in both directions (light blue) by passivating copper ions at higher concentrations and retaining nutritional access to trace copper at lower concentrations.

to phagolysosomes of macrophages implicates labile copper ions in phagocytic killing of *E. coli*. [122]. Consistent with a role for copper in phagolysosomal killing, Ybt-expressing UPEC exhibited a marked intracellular survival benefit following phagocytosis by macrophage-like RAW 264.7 cells [113,123]. While the protective effect of Ybt may be attributable to copper passivation, Cu(II)-Ybt was also observed to act as a superoxide dismutation catalyst, analogous to SOD, a remarkable activity for a nonprotein biomolecule. Given the importance of SOD to intracellular survival in pathogenic bacteria, this may be an additional virulence-associated activity connected to Ybt production in UPEC.

While Ybt-mediated copper uptake and passivation are seemingly contradictory functions, their co-existence may be an ingenious solution to a challenging biological problem. UPEC will secrete Ybt in molar excess of available copper, which could result in self-induced copper starvation. By permitting controlled Cu(II)-Ybt import, copper starvation is avoided. In the absence of copper toxicity, Ybt can act as a copper scavenger (a chalkophore) similarly to a siderophore. This dual functionality of Ybt Cu(II) binding activity has been termed nutritional passivation: a strategy of minimizing a metal ion's toxicity while preserving its nutritional availability (Figure 3) [112]. This flexibility of function may help UPEC adapt to multiple environments in the human urinary tract.

Escherichelin as an inhibitor of Pseudomonas siderophore-mediated iron uptake

A recent study found that Ybt is not the only specialized metabolite connected to the *Yersinia* HPI in UPEC [111]. A mass spectrometry-based metabolomic screen identified a prominent metabolite called escherichelin (Figure 2) as the product of a branch of the Ybt biosynthetic pathway. This specialized metabolite lacks the siderophore activity of Ybt, though it is capable of binding Fe(III). Surprisingly, escherichelin's structure matches that of a synthetic *Pseudomonas* antivirulence compound described by Mislin *et al.* in 2004 [124]. Escherichelin inhibits iron uptake by pyochelin, the structurally related, virulence-associated siderophore of *Pseudomonas* and related species. Inhibition appears to occur when escherichelin forms a nontransportable ferric complex with pyochelin in the binding pocket of the outer membrane receptor FptA [125]. As such, escherichelin may promote niche dominance during UTI pathogenesis, creating a less hospitable environment for *Pseudomonas* and other strains that use the pyochelin siderophore system. Although the pyochelin and Ybt biosynthetic pathways both generate the same escherichelin precursor, escherichelin production is greatly suppressed in *Pseudomonas* relative to UPEC, consistent with the expected species-specific selective pressure.

Production of an inhibitor of *Pseudomonas* virulence by urinary *E. coli* strains suggests a mechanistic reason for the positive association between antibiotic exposure (which would tend to suppress Ybt-producing *Enterobacteriaceae*)

and UTI caused by relatively antibiotic-resistant *Pseudomonas* species [126]. Indeed, therapeutic bladder colonization with an asymptomatic bacteriuria *E. coli* strain HU2117 [127] (a papG adhesion-null derivative of *E. coli* 83972) has been proposed to prevent *Pseudomonas* and other opportunistic infections. Mass spectrometric analysis of patients experimentally inoculated with HU2117 found abundant urinary escherichelin in successfully colonized patients [111]. These findings again expand the possible repertoire of siderophore system activities to include effectors of interspecies antagonism.

Conclusion

Together, these recent findings point to complex, nuanced mechanisms for both host and pathogen metal interactions. Metal ions are both nutritionally essential and potentially toxic to bacteria, resulting in sophisticated regulatory mechanisms in both host and pathogen. In fact, a number of UPEC virulence factors are involved in metal interactions – pointing to the critical importance of metal regulation during infection. Although originally regarded solely as part of a redundant iron acquisition system, recent findings support a distinctive and nonredundant metallophore function for Ybt. Mathematical analysis of the genetic distribution of virulence factors in UPEC indicates distinct communities of virulence factors, suggesting a variety of survival strategies. A parallel theme is observed when the complexity of UPEC metal interaction systems is considered in mechanistic detail – a range of mechanisms exist, each with unique benefits that are likely favorable under specific conditions. Within the diversity of microenvironments relevant to UTI pathogenesis, uropathogens likely benefit by drawing upon a repertoire of metal-interacting functions.

Future perspective

Mechanistic studies of bacterial metal uptake and resistance will continue to provide new insights into human–microbe interactions. These insights, in turn, will also suggest new anti-infective strategies based upon pharmaceutical agents, nutritional supplementation or probiotics. Progress in this area will be aided with an improved appreciation for which UPEC functions are redundant and which confer unique, virulence-associated properties. The answers to these questions may depend upon the specific combination of genes and polymorphisms present in a given UPEC isolate. A combination of functional and genomic studies may provide answers to these questions. The prospect of therapeutically manipulating the role of copper in UTI pathophysiology would benefit from

Executive summary

Uropathogenic *Escherichia coli*

- Antibiotic resistance and post-antibiotic recurrence challenge current treatment approaches.
- Uropathogenic *Escherichia coli* (UPEC) virulence factors are distributed in statistically linked groupings, each with a representative siderophore system.
- Siderophore expression is associated with UPEC isolates.

Metal ion control in the host

- Hosts control the location and complexed form of biological metal ions through systemic and local responses.
- These regulatory processes are enhanced during inflammation in a process termed nutritional immunity which results in the appearance of multiple iron sequestration proteins in the urinary tract during infection.
- Siderocalin (Lipocalin-2) sequesters iron either by binding ferric enterobactin or, in the urinary environment, by binding a ferric complex involving urinary catechol metabolites; in the latter case, the iron remains accessible to siderophores.

UPEC metal acquisition systems

- Overlapping systems in UPEC import metals as free ions, host-derived complexes and UPEC-derived siderophore complexes.
- Direct mass spectrometric detection in clinical specimens demonstrates UPEC siderophore production during urinary tract infection.
- The siderophore enterobactin liberates iron from siderocalin complexes during growth in human urine.

Noncanonical functions of the UPEC *Yersinia* high pathogenicity island

- UPEC uses yersiniabactin as a metallophore to bind, and mediate import of, both iron and non-iron metal ions.
- Yersiniabactin counteracts copper ion toxicity.
- The microbial strategy of minimizing a metal ion's toxicity while preserving its nutritional availability has been termed nutritional passivation.
- Escherichelin, a newly appreciated siderophore-like *Yersinia* high pathogenicity island product, is produced during clinical *E. coli* bacteriuria and inhibits siderophore-dependent *Pseudomonas* growth.

a more extensive understanding of mammalian copper homeostasis. We anticipate that additional examples of nutritional passivation will be identified in non-UPEC bacteria. Lastly, the means by which some bacteria interfere with transition metal acquisition by different bacterial species may be harnessed or mimicked in future probiotic or pharmacologic therapies [128].

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