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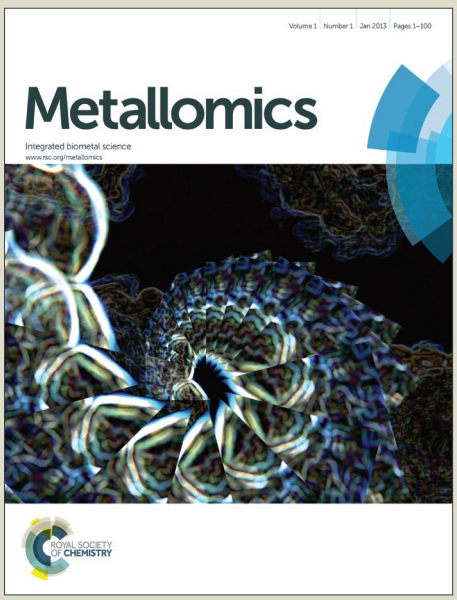

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Back to the Metal Age: Battle for Metals at the Host-Pathogen Interface During Urinary Tract Infection

Running Title: Battle for Metals During Urinary Tract Infection

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ABSTRACT

Urinary tract infection (UTI) represents one of the most common bacterial infections in humans and uropathogenic *E. coli* (UPEC) is the major causative agent of UTI in people. Research on UPEC and other bacterial pathogens causing UTI has now identified the critical role of metal transport systems in the pathogenesis of UTI. Here we review the major effectors of metal transport in bacteria and host proteins that impair metal acquisition by bacterial pathogens. In particular, we describe the studies that identified iron, zinc and nickel import and copper export as key virulence and fitness determinants during UTI. Various metal transport systems and mechanisms that govern the expression of metal transport systems are also presented here. Specific examples from UPEC and other uropathogens, when available, are presented to depict the battle for metals at the host-pathogen interface during UTI.

Urinary tract infection (UTI) is one of the most common bacterial infections and the most common reason for antibiotic prescription in humans.[1, 2] Uropathogenic *Escherichia coli* (UPEC) is the predominant cause of UTI in humans. In otherwise healthy individuals, 75-95% of UTIs are due to UPEC colonization in the urinary tract.[1] Other prominent causes of UTI include *Proteus mirabilis, Klebsiella pneumoniae, Enterobacter aerogenes, Citrobacter* species, *Providencia stuartii, Staphylococcus saprophyticus* and *Acinetobacter baumannii.*[1] UTI caused by pathogens other than UPEC are more common in peoplewith anatomical or neurological abnormalities in the urinary tract resulting in incomplete voiding, indwelling catheters or in elderly patients with underlying co-morbidities such as diabetes mellitus and immune dysfunction. Women are more highly predisposed to UTI than men, primarily due to anatomic differences in the urogenital tract.[1]

UTIs are usually ascending in nature, beginning with bacterial colonization and inflammation of the urinary bladder, known as cystitis.[3, 4] In most patients, uropathogens colonize the gut prior to a clinical episode of UTI. Cystitis is marked by painful, frequent voiding of small volumes of urine and may be accompanied by fever. In a subset of individuals with cystitis, UPEC ascends to the kidneys via the ureters resulting in inflammation of the renal pelvis and parenchyma, known as pyelonephritis. Pyelonephritis is usually accompanied by fever and flank pain, and requires immediate medical attention. Uncontrolled pyelonephritis, in some cases, results in potentially fatal complications such as bacteremia and sepsis. At the opposite end of these inflammatory events resulting from bacterial colonization is asymptomatic bacteriuria (ABU). Urinary

tracts of individuals with ABU are colonized by *E. coli* strains in the absence of symptoms typically associated with UTI.[5]

Bacterial pathogens utilize a diverse array of virulence mechanisms to reach the bladders and kidneys, adhere to epithelial cells, survive and continue to grow within the urinary tract and eventually subvert host defenses to successfully establish a UTI.[6-8] Metals such as iron, magnesium, manganese, nickel, zinc, and cobalt serve as cofactors for various critical enzymes in most forms of life, including bacteria.[9] Key virulence traits displayed by uropathogens include the ability to pilfer essential metals from host and to efflux toxic metals.

During infection, bacterial pathogens must acquire essential metals from the host and an intense competition for these metals ensues at the host-pathogen interface. Sequestering essential metals by the host impairs growth of pathogens *in vivo* and represents an attractive strategy to deter bacterial growth. Mammalian hosts produce high-affinity metal-binding proteins that limit bioavailability of free metals. For instance, lipocalin is a host protein that binds enterobactin, a bacterial iron-chelating molecule, and prevents enterobactin-mediated iron uptake. Host metal-binding proteins are effectors of nutritional immunity, an integral part of innate immune response to infection.[9] However, pathogens have evolved specific systems to counteract nutritional immunity effectors. Better understanding of nutritional immunity mechanisms involved in UTIcould offer novel insights to develop strategies to combat uropathogens, especially those that are recalcitrant to treatment with antibiotics. Among uropathogens, battle for the metals is

relatively well characterized for UPEC and is the major focus of this review. Specifically, we will discuss the importance of acquiring iron, nickel, and zinc, and efflux of copper during UTI. When available, pertinent examples fromother uropathogens are also discussed.

Omic Studies Elucidate the Involvement of Metal Transport During UTI

Global approaches utilizing omics technology have shed light on understanding the importance of metal ion transport systems in bacterial pathogens during UTI. Genomic, transcriptomic, proteomic, immunoproteomic and metabolomic studies have been utilized to elucidate the role of Fe uptake systems in the pathogenesis of UTI by UPEC. Sequencing genomes of multiple UPEC strains and molecular epidemiology studies have revealed a higher prevalence of salmochelin, yersiniabactin, aerobactin and heme receptors in UPEC, compared to fecal commensal strains of *E. coli*.[10-14] Multiple Fe uptake systems are among the most highly expressed genes in UPEC during UTI in patients and during experimental infection in a murine model (Table 1).[15-17] During intracellular growth, the heme receptor gene *chuA* is highly upregulated.[18] The majority of proteins identified in differential proteomic analysis of UPEC cultured in human urine *ex vivo* are involved in Fe acquisition.[19] Serum from mice with prior UTI (convalescent serum) recognizes multiple outer membrane Fe uptake proteins in UPEC.[20] Finally, direct measurement of siderophore levels in UTI urine samples revealed the presence of multiple siderophores during infection.[21] In summary, omics-enabled technology has elucidated the role of Fe uptake systems in various settings relevant to the biology of UPEC infection.

Genes involved in Cu^{1+}, Ni^{2+}, Zn^{2+}, and Mn^{2+} transport were highly upregulated in UPEC during infection (Table 1). Cu^{1+} efflux system genes are highly upregulated during human UTI compared to culture in urine *ex vivo.*[17] Specifically, the Cus system appears to be involved in Cu detoxification during acute UTI. Genes involved in Mn^{2+} and Fe^{2+} iron import, *sitABCD,* are highly expressed in urine and during infection.[15] Involvement of Fe^{2+}importers, if any, in the pathogenesis of UTI has not been reported. Recently, Ni^{2+} uptake system genes *nikABCD* were reported to be specifically expressed during human UTI.[17]

Microarray-based transcriptional profiling of *P. mirabilis,* cultured under Fe-limited conditions, revealed the genes involved in Fe uptake including siderophore systems, heme receptors and receptors for exogenous siderophores, in this uropathogen.[22] This study also led to the identification of proteobactin, a novel siderophore system, and a yersiniabactin-related siderophore system in *P. mirabilis.* Fe uptake system genes, including *sitABC, exbBD, hmuS, ireA* and *feoAB,* are highly expressed in *P. mirabilis* during experimental UTI in a mouse model (Table 1).[23] Fe uptake receptors (PMI 0842 and 2596), heme receptor HmuR2 and Zn uptake system protein ZnuB were identified as antigenic proteins in a immunoproteomic screen using convalescent serum from mice chronically infected with *P. mirabilis.*[24] In summary, comprehensive omic studies have guided hypothesis-driven research on the role of metal uptake systems during UTI in patients and in experimental infection models.

Iron Acquisition in Urinary Tract

Although Fe is among the most abundant metals in earth, bioavailability of Fe is extremely limited within mammalian hosts and Fe limitation represents a well known facet of nutritional immunity.[9] Since free Fe produces highly reactive and damaging hydroxyl radicals via the Fenton reaction, elemental Fe is conjugated to proteins during transport and storage. Glycoproteins such as transferrin and lactoferrin are used to transport Fe and Fe is incorporated into active sites of enzymes or in the heme moiety in myoglobin and hemoglobin found in myocytes and erythrocytes, respectively. Therefore, bacteria must use specialized systems to acquire Fe from the host.

Bacteria can import iron directly in the Fe^{2+} or Fe^{3+} form and indirectly by uptake of Fe-containing molecules such as heme and hemoglobin. A unifying theme in otherwise diverse Gram-negative bacterial Fe uptake systems is the involvement of TonB-ExbB-ExbD complex localized in the inner membrane. TonB is energized with proton motive force by ExbB and ExbD, and TonB transduces this energy to the Fe uptake receptors located on the outer membrane to facilitate translocation of Fe-containing molecules to the periplasm. Within the periplasm, cognate periplasmic-binding proteins bind to Fe-containing molecules. In the final step of transit, ABC (ATP-binding cassette) transporter-mediated ATP-dependent active transport is used to transport the cargo across the inner membrane. Given the central role of TonB in Fe acquisition, it was hypothesized that a UPEC *tonB* mutant would be attenuated during UTI. Indeed, the *tonB* mutant was highly attenuated in a mouse model of UTI underscoring the vital role of TonB-mediated Fe acquisition systems in the pathogenesis of UTI.[25]

To counteract high affinity Fe^{3+} iron chelators, known as siderophores, produced by bacteria, mammalian hosts produce siderophore-binding proteins to prevent reuptake of ferrisiderophores into bacterial cell. The best-illustrated example of this phenomenon is the binding of enterobactin, a siderophore, by lipocalin-2 (LCN-2), a siderophore-binding protein. Not surprisingly, uropathogenic bacteria such as UPEC and *K. pneumoniae* produce additional LCN-2 evading siderophores including a glycosylated variant of enterobactin (salmochelin), yersiniabactin and aerobactin.[21, 26] Indeed LCN-2 resistant siderophores are found more frequently in UPEC isolates, compared to fecal commensal *E. coli* isolates.[21, 27]

Regulation of Iron Homeostasis in Bacteria

The primary transcriptional regulator governing bacterial Fe uptake and metabolism is ferric uptake regulator (Fur). Fur is an Fe-sensing transcriptional repressor that is found in apo- and holo-form during low and high intracellular levels of Fe, respectively.[28] Members of Fur regulon include genes involved in uptake, storage and use of Fe. Holo-Fur binds with high-affinity to inverted repeats (Fur boxes) in the promoter region of Fur-regulated genes resulting in transcriptional repression. Apo-fur, however, has poor affinity for Fur boxes resulting in transcriptional derepression of Fe uptake genes during growth in iron-limited milieu such as the urinary tract and in urine *ex vivo*. A UPEC *fur* mutant is outcompeted by the wild-type strain during co-infection but is capable of colonization during independent infection in a UTI model.[29]

In addition to transcriptional regulation, small RNA-mediated post-transcriptional regulation is also part of the genetic regulatory circuit governing Fe homeostasis *E. coli.* Holo-Fur negatively regulates RyhB, a small regulatory RNA, that subsequently negatively regulates *acnA, ftnA, fumA*, and *sdhCDAB* transcripts whose products require Fe as a co-factor or are involved in Fe storage.[30] Loss of RyhB in UPEC results in attenuation in a UTI model and is linked to the reduced levels of siderophores secreted by the *ryhB* mutant strain.[29] Together, these regulatory circuits precisely activate or limit Fe import based on cellular demand.

Siderophore-mediated Iron Acquisition

Uropathogens produce siderophores to acquire the essential element Fe in Fe^{3+} form. Ferri-siderophore complexes are imported via cognate outer membrane receptor utilizing the energy transduced by the TonB-ExbB-ExbD complex (Fig. 1). UPEC may produce up to four siderophores: enterobactin, salmochelin, aerobactin, and yersiniabactin.[21] Genes involved in the biosynthesis and uptake of enterobactin are found in both UPEC and fecal commensal strains. However, biosynthetic and uptake machinery for salmochelin, aerobactin, and yersiniabactin are located on pathogenicity-associated islands typically found in UPEC, but not fecal commensal strains. Genomic localization to pathogenicity-associated islands suggests that these siderophore systems were acquired by horizontal gene transfer.[10, 11, 31]

Siderophores vary in structure and three major classes of siderophores are produced by UPEC.[32] Catechol group contains the coordination sites for Fe chelation in

enterobactin and salmochelin. Aerobactin and yersiniabactin contain a hydroxamate and heterocyclic ring as the coordination sites, respectively. Although Fe^{3+} chelation is the primary function of siderophores, recently non-Fe uptake functions have been described for siderophores in UPEC. Yersiniabactin binds Cu^{2+} and protects against cellular damage in UPEC (*See section on Copper Detoxification*).[33] Additionally, catecholate siderophore biosynthesis has been demonstrated to promote resistance against oxidative stress in *E. coli.*[34] Taken together, siderophores aid not only in Fe^{3+} acquisition but also confer resistance to copper toxicity and oxidative stress in UPEC.

Since gut colonization by UPEC precedes UTI, quantitative metabolomics was used to determine siderophore production in UPEC isolated from rectum and urine in UTI patients.[21] There was no discernable difference in enterobactin production between fecal and urine isolates. Urine isolates, however, produced significantly higher quantities of salmochelin and yersiniabactin compared to fecal isolates. These findings suggest that salmochelin and yersiniabactin, but not enterobactin, is involved in urofitness and are reminiscent of the role of LCN-2 during UTI (*see section on Nutritional Immunity*).[21]

Relative contribution of individual siderophores to fitness during UTI was assessed using UPEC mutants lacking a specific siderophore receptor. Ability to import aerobactin and yersiniabactin confers greater fitness advantage during UTI compared to hydroxamate or catecholate siderophore import.[35] These results are consistent with the role of LCN-2 in curbing ferric-enterobactin uptake and potential absence of hydroxamate siderophores, typically produced by fungi, within urinary tract. In a UPEC

isolate belonging to *E. coli* clonal group A, the catecholate siderophore receptor Iha functions as a fitness factor during UTI. These results suggest that strain-specific differences might exist in siderophore preference *in vivo* and possibly, Iha might be involved in adherence to urothelial cells during infection as demonstrated *in vitro*.[36] On the contrary, during asymptomatic colonization of murine urinary tract by *E. coli* strain 83972 catecholate siderophores, enterobactin and salmochelin, provide greater fitness advantage compared to aerobactin and yersiniabactin.[37] Complete reversal in siderophore preference between UPEC and asymptomatic bacteriuria strain during urinary tract colonization could be reconciled by the drastic difference in the outcome of colonization with these strains. UPEC strains induce a robust neutrophil-driven acute inflammatory response resulting in classic symptoms associated with UTI, while ABU strains cause asymptomatic colonization generally devoid of symptoms observed during UTI.

Compared to UPEC, Fe uptake mechanisms in *P. mirabilis* are less well characterized. *P. mirabilis* produces proteobactin and a yersiniabactin-related siderophore to acquire Fe^{3+} and the yersiniabactin-related siderophore contributes to successful colonization of urinary bladder.[22] The yersiniabactin-related siderophore was originally identified as a fitness gene in a signature-tagged mutagenesis screen designed to detect virulence factors in a murine UTI model.[38] Genomes of other uropathogens including *A. baumannii, Citrobacter* species, *Enterobacter aerogenes, K. pneumoniae, P. stuartii, S. aureus* and *S. saprophyticus* also harbor the genes capable of producing various siderophores. The contribution of these siderophores to virulence during UTI, however, remains to be evaluated.

Nutritional Immunity

LCN-2 binds specifically to enterobactin, which is produced by several bacteria including UPEC and *K. pneumoniae.*[26] Using a LCN-2 reporter mouse, renal medullary cells were demonstrated to produce LCN-2 in response to UPEC in a TLR4-NF-κB dependent pathway.[39] Recently, α-intercalated cells in the renal medulla were reported as the specific cellular source of LCN-2 found in urine during UTI caused by UPEC.[40] Additionally, LCN-2 is produced by the epithelial cells lining the urinary bladder, ureters and kidneys as well as neutrophils transmigrating into the urinary tract in response to bacterial colonization.[41] Mice lacking LCN-2 are highly susceptible to experimental UTI caused by UPEC indicating the protective role of LCN-2 during UTI.[40, 41] Specificity of LCN-2 to enterobactin is demonstrated by the finding that *E. coli* strains that are completely dependent on enterobactin for iron uptake can infect only LCN-2-deficient, but not wild-type mice.[40] Urinary LCN-2 levels are higher during naturally occurring UTI in humans and experimental UTI in murine models.[40, 41] Furthermore, supplementation of LCN-2 to urine *ex vivo*, impedes growth of UPEC by limiting Fe availability.[40] These studies highlight the importance of LCN-2, a key nutritional immunity effector in inhibiting enterobactin-mediated bacterial Fe uptake during UTI.

Hepcidin is a peptide hormone produced by hepatocytes in response to a range of stimuli, including bacterial infection. Hepcidin prevents efflux of Fe from hepatocytes into circulation and establishes transient systemic hypoferremia by binding to the iron transporter, ferroportin, and targeting it to degradation.[42] The role, if any, of hepcidin

during UTI has not been reported. Given the importance of bacterial iron uptake systems during UTI, it is likely that hepcidin plays a protective role against bacterial UTIs.

UTI urine samples contain more neutrophils, erythrocytes and epithelial cells as compared to urine from healthy subjects.[17] UPEC is endowed with cytolytic toxins, including hemolysin, and is potentially capable of releasing intracellular Fe and heme stores. Indeed, the total concentration of Fe in urine is higher during UTI (724 ± 185 nM) than healthy controls (161 ± 69 nM).[17] However, there is no significant difference in expression of Fe uptake genes between UPEC in patient urine samples and in urine from healthy volunteers, indicating that bioavailability of Fe could be restricted by LCN-2 and other proteins involved in nutritional immunity during UTI.[17]

Heme as an Iron Source

Pathogens can scavenge Fe from precursors such as heme and hemoglobin. UPEC contains two outer membrane heme receptors, ChuA and Hma.[43] Heme uptake via these receptors contributes to fitness during UTI; *hma* mutant outcompetes a *chuA* mutant indicating that ChuA is the predominant heme transporter during UTI.[43] ChuA is also involved in intracellular growth of UPEC in bladder epithelial cells. During intracellular growth, *chuA* is highly expressed and a *chuA* mutant fails to grow at wild-type level.[18] Heme uptake systems contribute to fitness during extracellular and intracellular growth in UPEC. *P. mirabilis* also encodes HmuR1 and HmuR2, outer membrane receptors that facilitate heme import. Loss of HmuR2 results in attenuation in both bladders and kidneys in the murine UTI model.[44] Heme uptake appears to be a fitness mechanism

conserved between UPEC and *P. mirabilis.*

Targeting Iron Acquisition Systems to Prevent UTI

Importance of Fe acquisition during infection in experimental models of UTI and during clinical UTI in humans has been unequivocally demonstrated. Therefore, Fe uptake systems represent an attractive target for development of prophylactics against UPEC. Outer membrane components, FyuA, Hma, Iha, IreA, IroN and IutA, of multiple Fe^{3+} import receptors have been tested as vaccine candidates and protection against experimental UTI was evaluated in a murine model.[45, 46] Vaccination with IreA protects against cystitis whereas Hma and FyuA vaccines are protective against pyelonephritis. Vaccination with aerobactin receptor IutA is protective against both cystitis and pyelonephritis. It would be of interest to test the synergy in protection, if any, against UPEC by a multivalent iron uptake receptor vaccine. Additionally, a recombinant protein expressing select domains of *E. coli* iron uptake receptors was tested as a candidate vaccine.[47] This vaccine provided protection against experimentally induced peritonitis in mice. While presence of anti-*E. coli* sIgA was confirmed in vaginal wash samples, utility of this vaccine against UTI remains to be established. Recently, small molecule inhibitors of TonB activity were identified in UPEC strain CFT073 using a high-throughput screen of a large compound library.[48] These molecules represent valuable resources to study TonB function and represent potential candidates for translational research.

Zinc Acquisition

Zn^{2+} is another essential element found at a limiting concentration within

mammalian hosts and is sequestered by effectors of nutritional immunity.[49] Two distinct Zn^{2+} import systems ZnuACB (ABC transporter) and ZupT (permease) are found in UPEC (Fig. 2).[49] While ZnuACB is a Zn-specific transporter, ZupT can transport cobalt, Fe^{2+} and Mn^{2+} in addition to Zn. In minimal medium, ZnuACB is required for wild-type levels of growth but growth of ZupT-deficient strain is indistinguishable from wild-type strain. In a murine model of UTI, *znuACB* mutant is compromised in fitness, however *zupT* is dispensable for fitness, indicating that Zn^{2+} uptake via ZnuACB system contributes to wild-type level of fitness.[50] The ZnuACB system also contributes to Zn uptake in *P. mirabilis* both *in vitro* and *in vivo*. A *znuC* mutant is impaired in growth during Zn^{2+} limitation and exhibits a fitness defect during UTI in a mouse model.[51] Overall, Zn^{2+} uptake by ZnuACB system is involved in urofitness in both UPEC and *P. mirabilis.*

Calprotectin, a protein predominantly found in neutrophils, is another effector of nutritional immunity. Calprotectin chelates Mn^{2+} and Zn^{2+}, both essential metals for optimal growth of bacteria.[49] S100A8 and A9 subunits of calprotectin were found at higher levels in the bladder and kidneys during experimental UTI.[52] Since neutrophils are the primary players in defense against bacterial UTI and calprotectin is induced during UTI, it was speculated that calprotectin would be involved in protection against UTI. Calprotectin-deficient and wild-type mice exhibit similar UPEC burden in urinary tract, indicating that calprotectin-mediated chelation of Mn^{2+} and Zn^{2+} might not affect pathogenesis of UTI, at least in this model.[52]

Nickel Uptake

Ni^{2+} is a cofactor for a number of enzymes including urease and dehydrogenases associated with the formate hydrogen lyase complex in bacteria. Ni^{2+} import is achieved via an ABC-transport system in UPEC (Fig. 2). A UPEC mutant lacking *nikABCDER* genes exhibits a fitness defect in the bladder, indicating that Ni^{2+} acquisition contributes to survival *in vivo*.[17] Since UPEC strains are typically urease-negative, it is likely that other Ni^{2+} requiring processes are critical for survival of UPEC within urinary tract. Since many Ni^{2+}-containing enzymes, including the formate hydrogen lyase complex-associated hydrogenases, are active under low-oxygen conditions, it is not unreasonable to predict that UPEC may encounter oxygen depletion during UTI.[17]

Urease is a Ni^{2+}-containing metalloenzyme and Ni^{2+} is indispensable for the catalytic activity of urease. Urease catalyzes conversion of urea to ammonia and carbon dioxide resulting in rapid alkalinization of the milieu.[53] High pH leads to precipitation of magnesium and calcium-containing compounds found in the urine causing cystalluria (presence of crystals in urine) and urolithiasis (formation of calculi in the urinary tract). Urease is found in *P. mirabilis* and *S. aureus,* and is a key contributor of virulence during UTI. In *P. mirabilis,* the direct role of Ni^{2+} import genes in UTI has not been reported. However, loss of urease activity significantly attenuates this pathogen during UTI.[54] Therefore, we hypothesize that deficiency in Ni^{2+} uptake will also adversely affect the ability of *P. mirabilis* to cause UTI. Direct evidence for the role of Ni uptake during uropathogenesis has been established for *S. aureus*. A *S. aureus* mutant lacking *nik* genes is defective in colonizing murine urinary tract.[55] This mutant is also defective in urease

activity and fails to induce crystal formation in urine *in vitro*. Since urease is integral to the virulence of *P. mirabilis* and *S. aureus*, it might be difficult to investigate the urease-independent roles for Ni^{2+} in these pathogens. In light of the recent findings in UPEC, Ni^{2+} uptake, dependent and independent of urease activity, appears to contribute to fitness during UTI.

Copper Detoxification

Cu and heme form the catalytic core of cytochrome *bo* terminal oxidase (CyoABCD) found in the inner membrane of *E. coli*. When cellular Cu exceeds normal levels, Cu, specifically cuprous form (Cu^{+}), acts as an extremely toxic biocide.[56] Cu^{+} can generate extremely reactive hydroxyl radicals via the Fenton reaction, damaging iron-sulfur clusters and inactivating dehydratases involved in the production of branched-chain amino acids. *E. coli* uses dedicated efflux systems CopA, CueO and CusCFBA (Fig. 3), and Cu-sensing regulatory proteins CueR and CusRS to maintain normal intracellular levels of copper.[57] Severity of Cu stress and oxygen availability determines which system is involved in Cu efflux during a specific condition.

CopA is a P-type ATPase that transports Cu^{+} from the cytoplasm to the periplasm and is active during low levels of Cu toxicity.[58] During moderate Cu^{+} stress, a Cu-sensing transcriptional regulator CueR activates transcription of both *copA* and *cueO*.[59] CueO is a periplasmic multicopper oxidase that oxidizes Cu^{+} (most toxic) to Cu^{2+} (less toxic).[60] Under extreme Cu^{+} toxicity and in low oxygen conditions, CusCFBA efflux system is activated by the cognate two-component regulatory system CusRS.[57] CusS is an inner

membrane-associated sensor kinase that phosphorylates CusR in response to extreme Cu^+ stress and phosphorylated CusR activates transcription of *cusCFBA* genes.[61] CusCFBA system pumps Cu^+ to the exterior across inner membrane, periplasm and outer membrane. These systems act in concert to protect *E. coli* against Cu^+ toxicity.[57]

An *E. coli copA* mutant is highly susceptible to intracellular killing and this phenotype is primarily dependent on ATP7A-mediated transport of Cu into the phagosome.[62] Transcriptome of UPEC obtained from patient samples, compared with culture in urine from healthy volunteers, revealed that Cu efflux system genes, especially the Cus system genes, are specifically expressed during UTI.[17] To understand the significance of this observation in the context of UTI, we measured Cu levels in urine samples. Cu is found at higher levels in the urine of patients with UTI (287 ± 77 nM), compared to healthy controls (59 ± 14 nM).[17] Furthermore, Cu supplementation in drinking water reduces UPEC burden in the bladders and urine of mice.[17] Taken together, these results suggest that Cu-mediated killing of UPEC is an innate immune mechanism aimed at preventing UPEC growth within urinary tract.

Changes in cellular levels of one metal may affect the concentration of other metals. An inverse relationship between presence of periplasmic multicopper oxidase CueO and cellular Fe levels has been appreciated in UPEC. A mutant lacking CueO is capable of acquiring Fe at a higher level, compared to wild-type strain and indeed exhibits a fitness advantage in a mouse model of UTI.[63] Differences in fitness phenotypes between *cusSRCFBA* and *cueO* mutants could be attributed, at least in part, to the

preferential use of Cu efflux systems in different microenvironments. Testing the Fe uptake potential of a *cusSRCFBA* mutant and assessing the fitness of mutants lacking individual and different combinations of Cu efflux systems can facilitate further scrutiny of these dichotomous observations.

In addition to specific efflux systems, yersiniabactin production confers an additional fitness advantage for UPEC isolates during Cu stress. Cupric-yersiniabactin complexes have been demonstrated in urine from UTI patients, indicating a biological role for this interaction during infection.[33] The contribution of Cu chelation *versus* the canonical role in Fe acquisition in UPEC fitness in an experimental model of UTI is yet to be determined. Recently, yersiniabactin was demonstrated to exhibit a superoxide dismutase-like activity and thereby imparting enhanced protection against oxidative stress in UPEC.[64] UPEC utilizes dedicated Cu efflux systems and co-opts a siderophore to mitigate toxic effects of copper during UTI.

Concluding Remarks

During UTI, host and bacteria engage in an intense battle to control access to essential metals. The ability to win this battle might tilt the balance in favor of pathogen or host, and determine the outcome of the war, that is, the ability of a pathogen to cause UTI. Several research groups, using multiple lines of investigation, have independently demonstrated that the ability to import Fe^{3+}, Zn^{2+}, and Ni^{2+}, and export Cu^{+} are critical for successful colonization of the urinary tract. It would be of great interest to the field to assess role of these metal transport systems in the pathogenesis of UTI by non-UPEC

uropathogens. Another interesting area would be to assess whether changes in metal homeostasis in pathogens during infection affect virulence, independent of growth impairment. Looking forward, the research community can capitalize on the knowledge gained in the biology of metal transport during UTI and embark on a path towards identifying novel therapeutic or prophylactic strategies that target metal transport in uropathogens.

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REFERENCES

1. B. Foxman, *Nat Rev Urol*, 2010, 7, 653-660.
2. K. Gupta, T. M. Hooton, K. G. Naber, B. Wullt, R. Colgan, L. G. Miller, G. J. Moran, L. E. Nicolle, R. Raz, A. J. Schaeffer and D. E. Soper, *Clin Infect Dis*, 2011, 52, e103-120.
3. J. B. Kaper, J. P. Nataro and H. L. Mobley, *Nat Rev Microbiol*, 2004, 2, 123-140.
4. S. Yamamoto, T. Tsukamoto, A. Terai, H. Kurazono, Y. Takeda and O. Yoshida, *J Urol*, 1997, 157, 1127-1129.
5. U. Lindberg, I. Claesson, L. A. Hanson and U. Jodal, *J Pediatrics*, 1978, 92, 194-199.
6. T. J. Wiles, R. R. Kulesus and M. A. Mulvey, *Exp Mol Pathol*, 2008, 85, 11-19.
7. K. E. Sivick and H. L. Mobley, *Infect Immun*, 2010, 78, 568-585.
8. T. J. Hannan, M. Totsika, K. J. Mansfield, K. H. Moore, M. A. Schembri and S. J. Hultgren, *FEMS Microbiol Rev*, 2012, 36, 616-648.
9. J. E. Cassat and E. P. Skaar, *Cell Host & Microbe*, 2013, 13, 509-519.
10. R. A. Welch, V. Burland, G. Plunkett, 3rd, P. Redford, P. Roesch, D. Rasko, E. L. Buckles, S. R. Liou, A. Boutin, J. Hackett, D. Stroud, G. F. Mayhew, D. J. Rose, S. Zhou, D. C. Schwartz, N. T. Perna, H. L. Mobley, M. S. Donnenberg and F. R. Blattner, *Proc. Natl. Acad. Sci. USA*, 2002, 99, 17020-17024.
11. E. Brzuszkiewicz, H. Bruggemann, H. Liesegang, M. Emmerth, T. Olschlager, G. Nagy, K. Albermann, C. Wagner, C. Buchrieser, L. Emody, G. Gottschalk, J. Hacker and U. Dobrindt, *Proc. Natl. Acad. Sci. USA*, 2006, 103, 12879-12884.
12. S. Subashchandrabose, T. H. Hazen, D. A. Rasko and H. L. Mobley, *Pathogens and Disease*, 2013, DOI: 10.1111/2049-632X.12059.
13. P. D. Vigil, A. E. Stapleton, J. R. Johnson, T. M. Hooton, A. P. Hodges, Y. He and H. L. Mobley, *mBio*, 2011, 2.
14. R. R. Spurbeck, P. C. Dinh, Jr., S. T. Walk, A. E. Stapleton, T. M. Hooton, L. K. Nolan, K. S. Kim, J. R. Johnson and H. L. Mobley, *Infect Immun*, 2012, 80, 4115-4122.
15. J. A. Snyder, B. J. Haugen, E. L. Buckles, C. V. Lockatell, D. E. Johnson, M. S. Donnenberg, R. A. Welch and H. L. Mobley, *Infect Immun*, 2004, 72, 6373-6381.
16. E. C. Hagan, A. L. Lloyd, D. A. Rasko, G. J. Faerber and H. L. Mobley, *PLoS Pathogens*, 2010, 6, e1001187.
17. S. Subashchandrabose S, T. H. Hazen, A. R. Brumbaugh, S. D. Himpsl SD, S. N. Smith, R. D. Ernst, D. A. Rasko, H. L. Mobley. 2014. *Proc. Natl. Acad. Sci. USA.* doi: 10.1073/pnas.1415959112.
18. C. S. Reigstad, S. J. Hultgren and J. I. Gordon, *J Biol Chem*, 2007, 282, 21259-21267.
19. C. J. Alteri and H. L. Mobley, *Infect Immun*, 2007, 75, 2679-2688.
20. E. C. Hagan and H. L. Mobley, *Infect Immun*, 2007, 75, 3941-3949.
21. J. P. Henderson, J. R. Crowley, J. S. Pinkner, J. N. Walker, P. Tsukayama, W. E. Stamm, T. M. Hooton and S. J. Hultgren, *PLoS Pathogens*, 2009, 5, e1000305.
22. S. D. Himpsl, M. M. Pearson, C. J. Arewang, T. D. Nusca, D. H. Sherman and H. L. Mobley, *Mol Microbiol*, 78, 138-157.

23. M. M. Pearson, A. Yep, S. N. Smith and H. L. Mobley, *Infect Immun*, 79, 2619-2631.
24. G. R. Nielubowicz, S. N. Smith and H. L. Mobley, *Infect Immun*, 2008, 76, 4222-4231.
25. A. G. Torres, P. Redford, R. A. Welch and S. M. Payne, *Infect Immun*, 2001, 69, 6179-6185.
26. M. A. Bachman, V. L. Miller and J. N. Weiser, *PLoS Pathogens*, 2009, 5, e1000622.
27. P. D. Vigil, A. E. Stapleton, J. R. Johnson, T. M. Hooton, A. P. Hodges, Y. He and H. L. Mobley, *mBio*, 2011, 2, e00066-00011.
28. J. W. Lee and J. D. Helmann, *Biometals*, 2007, 20, 485-499.
29. G. Porcheron, R. Habib, S. Houle, M. Caza, F. Lepine, F. Daigle, E. Masse and C. M. Dozois, *Infect Immun*, 2014, 82, 5056-5068.
30. E. Masse and S. Gottesman, *Proc. Natl. Acad. Sci. USA*, 2002, 99, 4620-4625.
31. A. L. Lloyd, D. A. Rasko and H. L. Mobley, *J Bacteriol*, 2007, 189, 3532-3546.
32. A. Garenaux, M. Caza and C. M. Dozois, *Vet. Microbiol*, 2011, 153, 89-98.
33. K. S. Chaturvedi, C. S. Hung, J. R. Crowley, A. E. Stapleton and J. P. Henderson, *Nat Chem Biol*, 2012, 8, 731-736.
34. M. E. Achard, K. W. Chen, M. J. Sweet, R. E. Watts, K. Schroder, M. A. Schembri and A. G. McEwan, *Biochem J*, 2013, 454, 543-549.
35. E. C. Garcia, A. R. Brumbaugh and H. L. Mobley, *Infect Immun*, 2011, 79, 1225-1235.
36. S. Leveille, M. Caza, J. R. Johnson, C. Clabots, M. Sabri and C. M. Dozois, *Infect Immun*, 2006, 74, 3427-3436.
37. R. E. Watts, M. Totsika, V. L. Challinor, A. N. Mabbett, G. C. Ulett, J. J. De Voss and M. A. Schembri, *Infect Immun*, 2012, 80, 333-344.
38. L. S. Burall, J. M. Harro, X. Li, C. V. Lockatell, S. D. Himpsl, J. R. Hebel, D. E. Johnson and H. L. Mobley, *Infect Immun*, 2004, 72, 2922-2938.
39. N. Paragas, A. Qiu, Q. Zhang, B. Samstein, S. X. Deng, K. M. Schmidt-Ott, M. Viltard, W. Yu, C. S. Forster, G. Gong, Y. Liu, R. Kulkarni, K. Mori, A. Kalandadze, A. J. Ratner, P. Devarajan, D. W. Landry, V. D'Agati, C. S. Lin and J. Barasch, *Nat Medicine*, 2011, 17, 216-222.
40. N. Paragas, R. Kulkarni, M. Werth, K. M. Schmidt-Ott, C. Forster, R. Deng, Q. Zhang, E. Singer, A. D. Klose, T. H. Shen, K. P. Francis, S. Ray, S. Vijayakumar, S. Seward, M. E. Bovino, K. Xu, Y. Takabe, F. E. Amaral, S. Mohan, R. Wax, K. Corbin, S. Sanna-Cherchi, K. Mori, L. Johnson, T. Nickolas, V. D'Agati, C. S. Lin, A. Qiu, Q. Al-Awqati, A. J. Ratner and J. Barasch, *J Clin Invest*, 2014, 124, 5521.
41. M. Steigedal, A. Marstad, M. Haug, J. K. Damas, R. K. Strong, P. L. Roberts, S. D. Himpsl, A. Stapleton, T. M. Hooton, H. L. Mobley, T. R. Hawn and T. H. Flo, *J Immunol*, 2014, DOI: 10.4049/jimmunol.1401528.
42. E. Nemeth, M. S. Tuttle, J. Powelson, M. B. Vaughn, A. Donovan, D. M. Ward, T. Ganz, and J. Kaplan, *Science*, 2004, doi: 10.1126/science.1104742.
43. E. C. Hagan and H. L. Mobley, *Mol Microbiol*, 2009, 71, 79-91.
44. A. Lima, P. Zunino, B. D'Alessandro and C. Piccini, *J Med Microbiol*, 2007, 56, 1600-1607.

45. C. J. Alteri, E. C. Hagan, K. E. Sivick, S. N. Smith and H. L. Mobley, *PLoS Pathogens*, 2009, 5, e1000586.
46. A. R. Brumbaugh, S. N. Smith and H. L. Mobley, *Infect Immun*, 2013, 81, 3309-3316.
47. A. Wieser, E. Romann, G. Magistro, C. Hoffmann, D. Norenberg, K. Weinert and S. Schubert, *Infect Immun*, 2010, 78, 3432-3442.
48. A. Yep, T. McQuade, P. Kirchhoff, M. Larsen and H. L. Mobley, *mBio*, 2014, 5, e01089-01013.
49. T. E. Kehl-Fie and E. P. Skaar, *Curr Opin Chem Biol*, 2010, 14, 218-224.
50. M. Sabri, S. Houle and C. M. Dozois, *Infect Immun*, 2009, 77, 1155-1164.
51. G. R. Nielubowicz, S. N. Smith and H. L. Mobley, *Infect Immun*, 78, 2823-2833.
52. M. C. Dessing, L. M. Butter, G. J. Teske, N. Claessen, C. M. van der Loos, T. Vogl, J. Roth, T. van der Poll, S. Florquin and J. C. Leemans, *PloS one*, 2010, 5, e13394.
53. H. L. Mobley, M. D. Island and R. P. Hausinger, *Microbiol Rev*, 1995, 59, 451-480.
54. D. E. Johnson, R. G. Russell, C. V. Lockatell, J. C. Zulty, J. W. Warren and H. L. Mobley, *Infect Immun*, 1993, 61, 2748-2754.
55. A. Hiron, B. Posteraro, M. Carriere, L. Remy, C. Delporte, M. La Sorda, M. Sanguinetti, V. Juillard and E. Borezee-Durant, *Mol Microbiol*, 2010, 77, 1246-1260.
56. D. H. Nies and M. Herzberg, *Mol Microbiol*, 2013, 87, 447-454.
57. F. W. Outten, D. L. Huffman, J. A. Hale and T. V. O'Halloran, *J Biol Chem*, 2001, 276, 30670-30677.
58. C. Rensing, B. Fan, R. Sharma, B. Mitra and B. P. Rosen, *Proc. Natl. Acad. Sci. USA*, 2000, 97, 652-656.
59. F. W. Outten, C. E. Outten, J. Hale and T. V. O'Halloran, *J Biol Chem*, 2000, 275, 31024-31029.
60. G. Grass and C. Rensing, *J. Bacteriol*, 2001, 183, 2145-2147.
61. G. P. Munson, D. L. Lam, F. W. Outten and T. V. O'Halloran, *J. Bacteriol*, 2000, 182, 5864-5871.
62. C. White, J. Lee, T. Kambe, K. Fritsche and M. J. Petris, *J Biol Chem*, 2009, 284, 33949-33956.
63. J. J. Tree, G. C. Ulett, C. L. Ong, D. J. Trott, A. G. McEwan and M. A. Schembri, *J Bacteriol*, 2008, 190, 6909-6912.
64. K. S. Chaturvedi, C. S. Hung, D. E. Giblin, S. Urushidani, A. M. Austin, M. C. Dinauer and J. P. Henderson, *ACS Chemical Biology*, 2014, 9, 551-561.

Figure Legends

Graphical Abstract Legend

Uropathogenic *Escherichia coli* (UPEC) may produce enterobactin (green circles), salmochelin (green squares), aerobactin (magenta triangles) and yersiniabactin (purple ovals) during colonization of urinary tract. Epithelial cells and neutrophils produce and secrete (arrows) lipocalin (red rings) and calprotectin (purple circles) in response to bacterial urinary tract infection (UTI). Lipocalin chelates enterobactin to prevent reuptake of ferri-enterobactin complexes. However, salmochelin, aerobactin and yersiniabactin are available to compensate for the loss of enterobactin-mediated iton acquisition. Calprotectin is known to chelate manganese and zinc, but its role in UTI is not completely understood. Ceruplasmin (brown circles), a major copper-containing protein, is also found at epithelial cell-UPEC interface and might act as a source of copper to kill UPEC. Arrows indicate the direction of transport, import of Fe, Zn and Ni, and export of Cu, in UPEC during UTI.

Fig. 1. Enterobactin-mediated iron uptake in UPEC. Enterobactin-mediated iron uptake system is depicted as a model for TonB-dependent iron uptake systems in UPEC. Ferri-enterobactin complexes are transported across the outer membrane through FepA using the energy transduced by the TonB-ExbB-ExbD complex. A periplasmic-binding protein (FepB) delivers ferri-enterobactin to FepGD complex localized in the inner membrane. FepC is an ATPase that delivers energy for translocation of ferri-enterobactin across the inner membrane. UPEC, uropathogenic *E. coli*; OM, outer membrane; P, periplasm; IM, inner membrane; and C, cytoplasm.

Fig. 2. Import of Nickel and Zinc in UPEC. Ni^{2+} and Zn^{2+} are transported across the outer membrane by porins or as yet unidentified receptors. NikA is a periplasmic-binding protein that delivers Ni^{2+} to NikBC complex localized in the inner membrane. NikDE are ATPases that deliver energy for translocation of Ni^{2+} across the inner membrane. ZnuA is a periplasmic-binding protein that delivers Zn^{2+} to ZnuB complex localized in the inner membrane. ZnuC is an ATPase that delivers energy for translocation of Ni^{2+} across the inner membrane. Additionally, ZupT can also translocate Zn^{2+} across the inner membrane. UPEC, uropathogenic *E. coli*; OM, outer membrane; P, periplasm; IM, inner membrane; and C, cytoplasm.

Fig. 3. Copper transport in UPEC. Transport of Cu^{+} and Cu^{2+} across the outer and inner membrane is not clearly understood. CopA is an inner membrane-localized P-type ATPase that translocates Cu^{+} from the cytoplasm to the periplasm. CueO is a preiplasmic multicopper oxidase that converts more toxic Cu^{+} to relatively less toxic Cu^{2+}. CusABC complex forms an RND-type efflux pump that traverses both membranes and facilitates efflux of cytoplasmic Cu^{+} directly to the exterior. CusF is a periplasmic Cu^{+}-binding protein that delivers Cu^{+} to the CusABC complex. UPEC, uropathogenic *E. coli*; OM, outer membrane; P, periplasm; IM, inner membrane; and C, cytoplasm.

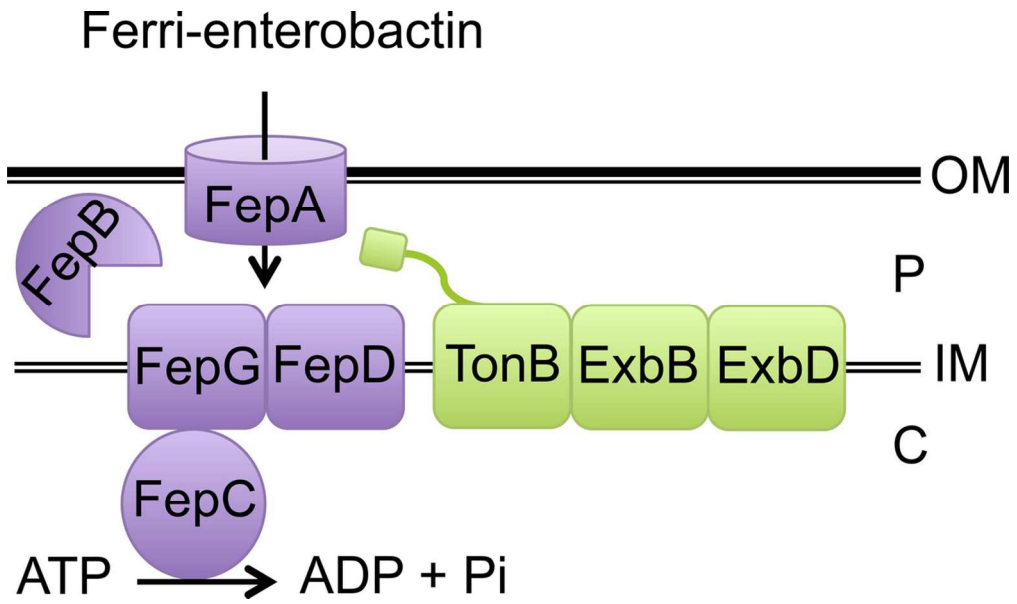
Table 1. Expression of metal transport system genes during UTI

Pathogen/ Metal[a]	Mouse model of UTI[b]	Human UTI[c]
UPEC		
Iron Uptake		
fepA	+	+
iroN	+	+
iutA	+	+
fyuA	+	+
chuA	+	+
hma	+	+
sitA	+	+
Other Metals		
nikA (Ni^{2+})	N	+
cusC (Cu^{2+})	N	+
P. mirabilis		
Iron Uptake		
exbB	+	N
exbD	+	N
sitA	+	N
hmuS	+	N
ireA	+	N
feoA	+	N

[a]Representative genes from up-regulated metal transport systems are indicated; UPEC, uropathogenic *E. coli.*

[b]N, not known; based on references 15, 18 and 23

[c]N, not known; based on references 16 and 17



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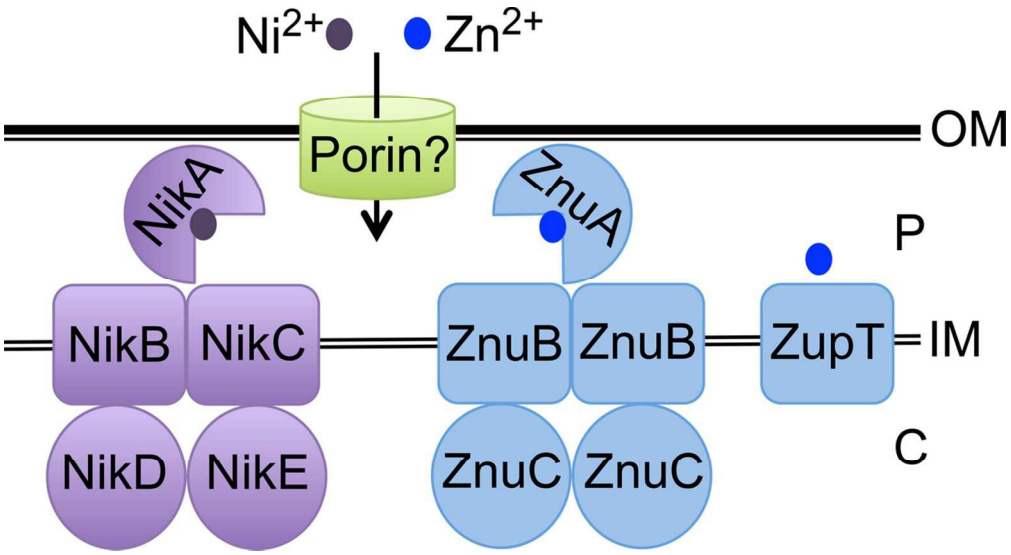

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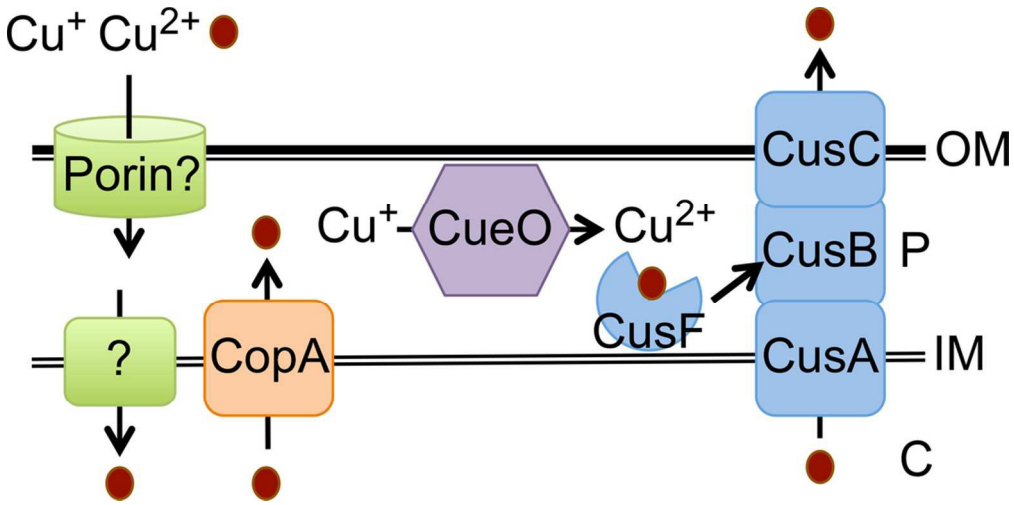

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