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Critical Review

Metal homeostasis in pathogenic Epsilonproteobacteria: mechanisms of acquisition, efflux, and regulation

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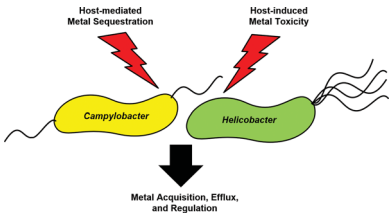
Abstract

Epsilonproteobacteria are a diverse class of eubacteria within the Proteobacteria phylum that includes environmental sulfur-reducing bacteria and the human pathogens, *Campylobacter jejuni* and *Helicobacter pylori*. These pathogens infect and proliferate within the gastrointestinal tracts of multiple animal hosts, including humans, and cause a variety of disease outcomes. While infection of these hosts provides nutrients for the pathogenic Epsilonproteobacteria, many hosts have evolved a variety of strategies to either sequester metals from the invading pathogen or exploit the toxicity of metals and drive their accumulation as an antimicrobial strategy. As a result, *C. jejuni* and *H. pylori* have developed mechanisms to sense changes in metal availability and regulate their physiology in order to respond to either metal limitation or accumulation. In this review, we will discuss the challenges of metal availability at the host–pathogen interface during infection with *C. jejuni* and *H. pylori* and describe what is currently known about how these organisms alter their gene expression and/or deploy bacterial virulence factors in response to these environments.

Keywords: Epsilonproteobacteria, metal, pathogen, *Campylobacter*, *Helicobacter*

Graphical abstract



This comprehensive review of metal acquisition systems and corresponding regulatory mechanisms provides insight into the importance of nutrient metal homeostasis to the survival of pathogenic Epsilonproteobacteria.

Introduction

Epsilonproteobacteria are a diverse group of flagellated microorganisms that have evolved into two distinct subgroups based on the environmental niche they inhabit.[1] The first subgroup, Nautiliales, includes oceanic thermophiles that are estimated to comprise 66–98% of the microorganisms inhabiting hydrothermal ocean vents and the surrounding area.[2] The second subgroup, and the focus of this review, consists of the clinically important Epsilonproteobacteria, *Campylobacter* spp. and *Helicobacter* spp., both of which have far-reaching impacts on global health (Fig. 1). Interestingly, *Helicobacter pylori* was initially considered to be a member of the *Campylobacter* genus,[3] but has since become recognized as a unique genus. Despite the genetic relatedness of *Campylobacter* and *Helicobacter*, and the ability of both to cause gastrointestinal disease, the pathogens have diverged to colonize different niches within the gastrointestinal tract and may encounter differing host responses. To combat these responses and successfully colonize, both *Campylobacter* and *Helicobacter* have evolved mechanisms to maintain appropriate levels of transition metals from the host environment with some systems being shared and others differing between these two pathogens.

Campylobacter spp. are one of the most prevalent causes of bacterial-derived gastroenteritis worldwide[4,5] with *Campylobacter jejuni* being responsible for ~90% of human campylobacteriosis cases and *Campylobacter coli* being responsible for ~10%.[6] As a result, *C. jejuni* is responsible for up to 15% of global cases of diarrhea with an estimated 1.3 million annual cases in the USA.[5,7,8] In the developed world, *Campylobacter* spp. are most often acquired through the consumption of improperly prepared or cross-contaminated food[9] primarily due to *Campylobacter*'s ability to asymptomatically reside within the gastrointestinal tract of

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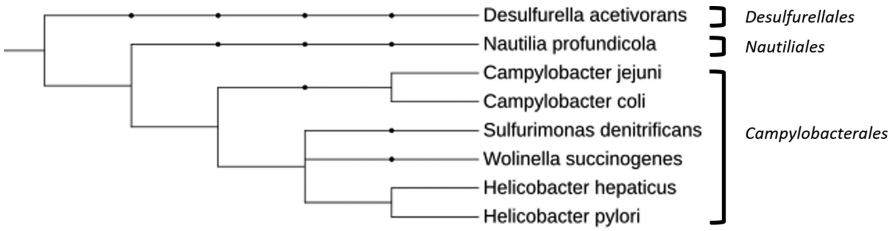

Fig. 1 Phylogeny of Epsilonproteobacteria. An example phylogenetic tree of Epsilonproteobacteria with example members from the Campylobacterales and Nautiliales orders. A Deltaproteobacteria (*Desulfurella acetivorans*) has been included as an example from another class. *Campylobacter* and *Helicobacter* belong to the same order, but branch into different families with different host niches. Adapted from Waite *et al.*[233] and visualized with iTol.[234]

agricultural animals, including chickens, cows, pigs, and sheep.[10–12] In contrast, the most common source of infection in the developing world is believed to be contaminated water.[13] Following ingestion of relatively few viable *Campylobacter* cells, the organism adheres to the intestinal mucosa and causes inflammation and inhibition of fluid reabsorption.[14–17] The pathogen has also been shown to invade host epithelial cells and can survive intracellularly, but campylobacters are generally considered extracellular pathogens[18] and are treated as such in colonization studies described in this review, unless otherwise stated. Colonization of a susceptible host typically results in a mild-to-moderate, self-limiting diarrhea that may contain blood, though several persistent post-infectious disorders can occur, including Guillain–Barré syndrome, irritable bowel syndrome, and reactive arthritis.[8] Due to this prevalence and severity of infection, both the Centers for Disease Control and Prevention and the World Health Organization have declared *Campylobacter* a severe threat to global health.[9]

The *Helicobacter* group contains several members, including gastric and non-gastric bacteria, but the most impactful, and a focus of this review, is the pathogen *H. pylori*.[19] Similar to *C. jejuni*, instances of infection and persistent colonization are greater in developing countries, with an estimated 80% of individuals being persistently colonized.[19,20] Colonization typically occurs at an early age and persists indefinitely with *H. pylori* becoming a primary constituent of the gastric microbiota unless the individual undergoes antibiotic treatment.[21] Nearly all individuals infected with *H. pylori* will experience chronic gastritis; however, a small proportion will develop more severe disease such as peptic and duodenal ulcers, neoplasia, hyperplasia, dysplasia, B-cell lymphoma of mucosa-associated lymphoid tissue, and invasive gastric cancer.[21] While animal hosts serve as the primary reservoir for *Campylobacter* leading to human disease, *H. pylori* lacks an environmental host and is instead thought to be passed between individuals through direct contact.[19] Studies have demonstrated the potential of *H. pylori* to survive intracellularly in host gastric cells, but it was initially considered an extracellular pathogen[22] and the systems addressed in this review have been described in extracellular *H. pylori* unless otherwise stated. *Helicobacter pylori* is the dominant prokaryote in the gastric niche, and the gastric environment provides numerous nutritional opportunities and challenges, including changes in osmolarity, low pH, innate immune antimicrobial onslaught, and alterations in metal cation concentrations.[23–26]

Transition metals are vital for all living cells as these ions participate in a variety of critical biological processes, including incorporation into metalloproteins required for respiration, DNA replication, and central metabolism.[27–29] To limit growth and dissemination of invading pathogens, the host employs several strategies to bind and sequester essential metals like iron, nickel, and zinc with host proteins like S100A12 in a method termed nutritional immunity.[30] In contrast, the host can also use localized increases in metals like copper and bismuth as an antimicrobial strategy due to the toxic effects of elevated metal concentrations on invading pathogens.[31] To combat host nutritional immunity or accumulation of metals at antimicrobial levels, *C. jejuni* and *H. pylori* have evolved mechanisms to sense changes in metal availability and subsequently alter their physiology to maintain the homeostasis of various metals when invading a susceptible host. The purpose of this review is to briefly examine what is known about the metal challenges at the host–pathogen interface during *Campylobacter* and *Helicobacter* infection and what strategies and regulatory systems these bacteria use to counteract those pressures.

Iron

Iron serves a vital role in microbial growth as it is a required cofactor for many enzymes, a catalyst in electron transport processes, and necessary for cellular processes such as the citric acid cycle, DNA synthesis, respiration, and electron transfer.[32,33] In addition to the contribution of iron to growth, it can lead to the generation of reactive oxygen species in the presence of oxygen via the Haber–Weiss reaction, which can damage proteins, DNA, and lipids.[34–36] To avoid the generation of oxygen-derived free radicals[37] while maintaining iron for essential cellular processes, both animal hosts and bacteria have evolved mechanisms to tightly regulate iron concentrations. Due to nutritional immunity, iron levels within the host are also maintained well below the levels necessary for growth of Gram-negative bacteria.[38] For example, iron-binding proteins in the intestine are capable of sequestering free ferric iron to a concentration of 10^{-24} M, while most bacteria require at least 10^{-7} M iron for normal cell functions.[39] As a result of this host sequestration by lactoferrin and other proteins[40] and to avoid toxic levels of iron, Epsilonproteobacteria have evolved numerous mechanisms for the efficient uptake and regulation of cellular iron levels (Tables 1 and 2).

Campylobacter

Of the metals discussed in this review, the metalloregulation of iron in *Campylobacter* has been studied most extensively due to the canonical roles of iron in cellular processes such as DNA synthesis and electron transfer. In addition to the general requirement of iron as a cofactor for metalloenzymes described in other organisms, iron has also been implicated in acid stress tolerance in *C. jejuni*[41] and modulation of chemotaxis through Tlp2, both of which impact colonization potential.[42] As previously mentioned, since free iron concentrations are restricted in animal hosts due

Table 1. Acquisition and efflux mechanisms and regulation of these systems in *Campylobacter*. List of acquisition systems and the corresponding regulatory mechanism if known for each metal in *Campylobacter*. The asterisk denotes transport systems or regulatory mechanisms that have been experimentally tested *in vitro* and superscript A denotes systems investigated *in vivo*, while those with no superscript have been bioinformatically predicted or have not been experimentally examined

Metal	Intracellular condition	Transport systems (*Campylobacter*)	References	Regulation (*Campylobacter*)	References
Iron	High			*Fur	70, 71
				*PerR	70
	Low	*[A]ChuZABCD— heme	32, 50, 51	*Fur	70, 71
		*[A]CtuA/CfbpABC—transferrin	50, 54, 58	*[A]HeuR	8
		*CfrA/CeuBCDE—FeEnt	53, 50, 65		
		*[A]FeoB—ferrous iron	47		
		*P19	66, 67		
Zinc	High			Zinc conc.	101, 105
	Low	*[A]ZnuABC	101	PerR	101
				Fur	101
Copper	High	*CeuO—detox	137	*CosR/TatA1	138
		*CzcD—efflux	55	Copper conc.	66
	Low	*P19	66		
Nickel	High			*Nickel conc.	152
	Low	*NikZYXWV	152, 158, 160	*HydABCD	151, 160
Cadmium	High	*CzcABCD	194, 195	*[A]Cadmium conc.	189
		*Cj0612c	190		
	Low				
Cobalt, magnesium, and manganese	High	CzcD	55	Cobalt conc.	201
	Low	MntABC	105		
Bismuth, molybdenum, and tungsten	High			*[A]Bismuth conc.	219
	Low	*ModABC	223	*ModE	223, 224
		*TupABC	222, 223		

to nutritional immunity, *Campylobacter* relies on outcompeting hosts for iron or utilizing iron-associated host molecules as a nutrient source.[32] In the case of *C. jejuni*, several iron acquisition systems have been identified and characterized, including the use of free iron, heme, iron-containing glycoproteins, and siderophores.[43–45]

Free iron transporters allow pathogens to transport iron encountered during colonization.[46] Like the ferrous iron transporter identified in *Escherichia coli*, FeoAB in *Campylobacter* transports free ferrous iron across the periplasmic space and into the cytoplasm, especially under iron-limited conditions.[47] The FeoAB system is conserved across *C. jejuni* strains and is important for the persistence of *C. jejuni* after invasion into intestinal cells.[47] Interestingly, differences in colonization potential of *feoB* mutants in *C. jejuni* strains 11168, 43431, and 81–176 were observed in a chick colonization model, indicating potential differences in iron transport efficiency between strains encoding the same ferrous iron transport system.[47]

The majority of iron in animal hosts is complexed within heme, a molecule consisting of a porphyrin ring with a centrally located iron atom.[48,49] In order for a bacterium to use the iron contained within the heme complex, the heme must be transported into the cytosol via a membrane transport complex.[50] In *C. jejuni*, heme transport is thought to rely on the ChuZABCD system.[32] Upon initial binding by the heme receptor, ChuA, heme is translocated into the periplasm in a TonB-dependent manner.[45] The ChuBCD transporter then facilitates ATP-dependent movement of heme into the cytosol, but studies indicate transport can still occur without a functional ChuBCD.[50,51] Finally, hydrolysis of the porphyrin ring by the heme oxygenase (ChuZ) occurs to release the iron.[8,50] Previous transcriptomic work demonstrated *chuZABCD* was upregulated during colonization of the chicken cecum,[52] but no differences in colonization by a *chuA* mutant were observed, indicating that *Campylobacter* uses methods other than heme acquisition to obtain iron within the avian host.[8,53–55] Expression of *chuA* increased over 1000-fold during human infection studies, supporting a role for heme as an important source of iron during colonization of a human host.[56]

The use of host-derived iron-containing glycoproteins, like lactoferrin and transferrin, as iron sources has been described for several pathogens, including those in the Pseudomonadaceae, Pasteurellaceae, Neisseriaceae, and Moraxellaceae families.[57–59] It was initially believed that *Campylobacter* was unable to utilize these glycoproteins,[58] but it has since been demonstrated that *Campylobacter* possesses a transferrin receptor (CtuA) and transporter (CfbpABC), which allows for the use of transferrin as an iron source under iron-limited conditions[50,54,58] (Fig. 2). A *ctuA* mutant exhibited a significant decrease in colonization potential in chickens[60] and a somewhat reduced colonization potential in a rabbit model when compared to wild-type *C. jejuni*,[54,60,61] with transcription of both CtuA and CfbpABC upregulated in an iron-limited environment,[33,55] indicating transferrin as a potential source of iron during infection.

Fungi and Gram-negative bacteria commonly utilize an iron sequestration process that involves the energy-dependent transport of iron chelators, termed siderophores.[62] Importantly, *Campylobacter* does not encode genes to synthesize siderophores, but it can use exogenous siderophores produced by other microorganisms including those produced by the host gastrointestinal microbiome.[32,38] For example, *C. jejuni* has the ability to take up

Table 2. Acquisition and efflux mechanisms and regulation of these systems in *Helicobacter*. List of acquisition systems and the corresponding regulatory mechanisms if known for each metal in *Helicobacter*. The asterisk denotes transport systems or regulatory mechanisms that have been experimentally tested *in vitro* and superscript A denotes systems investigated *in vivo*, while those with no superscript have been bioinformatically predicted or have not been experimentally examined

Metal	Intracellular condition	Transport systems (*Helicobacter*)	References	Regulation (*Helicobacter*)	References
Iron	High			*[A]Fur	84, 85, 86
				Pfr	83
	Low	*[A]FeoB—ferrous iron	80	*[A]Fur	84, 85, 86
		FecAB—ferric dicitrate	81		
		FrpB—FeEnt	82		
Zinc	High	CadA—efflux	118	*Zinc conc.	25, 114
		CzcB—efflux	118		
		*[A]CznABC—efflux	117		
	Low			*Fur	83
Copper	High	*CopAP—efflux	118	*CopP	144
		*CrdAB—efflux	118	*CrdRS	143
		*CzcAB—efflux		*Fur	83
	Low				
Nickel	High	*Hpn—sequestration	174	*[A]NikR	175, 176
		*HspA—sequestration	174	Nickel conc.	173
		*[A]CznABC—efflux	117		
	Low	*[A]NiuBDE	183		
Cadmium	High	*CadA—ATPase	196, 197	*Cadmium conc.	196, 117, 174
		CznABC—efflux	117		
		*Hpn—sequestration	174		
		*HspA—sequestration	174		
	Low				
Cobalt, magnesium, and manganese	High	*CadA	196	*Pfr	218
				Cobalt conc.	196
	Low	*FecDE	182		
		*CeuE	182		
		*NiuBDE	183		
		*NixA	213		
		*CorA	217		
Bismuth, molybdenum, and tungsten	High			*Bismuth conc.	229
	Low	ModD	230		

the siderophore enterobactin (Ent)[53,63] using ferric–enterobactin (FeEnt) uptake systems.[32,64] To transport FeEnt across the outer membrane, *Campylobacter* encodes either one or both of the TonB-dependent receptors, CfrA[53] and CfrB.[65] *Campylobacter jejuni* then uses the CeuBCDE transport system to translocate FeEnt into the cytosol, where it is hydrolyzed through an unknown mechanism releasing iron into a usable form[50] (Fig. 2). In addition, a high-affinity iron transporter protein (P19) and a co-localized iron transporter (CJJ81176_1649–1655) are induced during periods of iron limitation and required for optimal growth in media supplemented with human fecal extracts, suggesting a dependence on these transporters in a nutrient-limited environment like the human gut.[66,67] Together, these systems represent conserved and effective strategies used by *Campylobacter* to acquire iron and infect susceptible hosts.[38,60,65,68,69]

Iron homeostasis must be tightly regulated to ensure adequate amounts are present for biological processes while avoiding toxic levels. As a result, many iron acquisition systems in *Campylobacter* are controlled by both positive and negative regulators. To prevent excess intracellular iron and respond to oxidative stress, *C. jejuni* possesses two iron-activated members of the ferric uptake regulator (Fur) family of transcriptional regulators, Fur and PerR (Fig. 2). These regulators are responsible for controlling iron homeostasis and the response to peroxide stress, respectively.[70] *Campylobacter jejuni* Fur is homologous to those of other Gram-negative organisms, functioning primarily as an iron-responsive transcriptional regulator and repressing transcription of iron uptake systems when intracellular levels of iron are high; however, *C. jejuni* Fur can also act as a transcriptional activator and in an iron-dependent manner.[7,71] Interestingly, the role of PerR as a sensor for oxidative stress in *Campylobacter* is unique among Gram-negative organisms because it uses intracellular iron levels to detect oxidative stress.[70] Since *C. jejuni* is a microaerophile, survival under aerobic conditions necessitates the concerted control of oxidative stress response systems,[72] including iron cofactored superoxide dismutase and peroxidases, and heme cofactored catalase.[72] Thus, the importance of Fur and PerR regulation, directly, indirectly, or co-dependently, is not limited to nutrient iron acquisition, but to environmental persistence and survival.[47,60,72–75]

Transcriptomic studies have determined the number of genes controlled by Fur or PerR can be as high as 200–300,[70,67] which suggests iron homeostasis is particularly important in *Campylobacter*.[32,60,76] Additionally, transcriptomic analysis of a *C. jejuni* strain mutated for a putative regulatory protein, subsequently termed

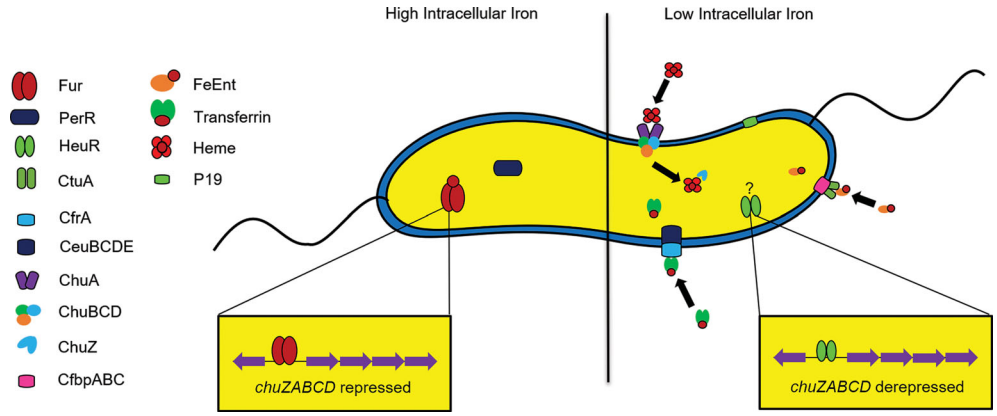

Fig. 2 Metalloregulatory mechanisms expressed during high and low intracellular iron concentrations in *Campylobacter*. High levels of intracellular iron induce activity of the holo (iron-bound) form of transcriptional regulators Fur and PerR. Low levels of intracellular iron result in the apo form of these regulators and the derepression of genes previously repressed. Low iron also induces increased expression of transporters for the uptake of iron molecules, including ferric–enterobactin (CtuA), transferrin (CeuBCDE/CfrA), and heme (ChuZABCD). Positive regulators of these systems, such as HeuR, also become active under iron-limited conditions.

HeuR (heme utilization regulator), identified the protein as a positive regulator of *chuZABCD* expression and other iron acquisition systems[8] (Fig. 2). This mutant exhibited decreased colonization of the chicken gut when compared to wild-type *C. jejuni* and was unable to utilize heme as a source of iron in an iron-restricted environment.[8] Since heme utilization has been shown not to be involved in chicken colonization, the reduction in colonization potential may be due to HeuR regulating other iron homeostasis systems; however, interactions between HeuR and Fur, which negatively regulates *chuZABCD* promoter activity, are currently unknown.[32,60,70,76]

Helicobacter

Helicobacter pylori also has a strict nutritional requirement for iron.[77] Within the gastric niche, *H. pylori* competes with the vertebrate host for dietary iron and has evolved the ability to utilize alternate sources of nutrient iron, including hemoglobin, heme, transferrin, and lactoferrin.[78,79] Because of this nutritional flexibility and changes in iron availability within the gastrointestinal tract, *H. pylori* has evolved elegant strategies to sense and respond to changes in iron concentrations. *Helicobacter pylori* encodes several iron transporters, including a homolog of the ferrous iron transporter FeoB found in *Campylobacter* and *E. coli*, although no homolog to FeoA has been identified.[80] Expression of a functional FeoB has been found to be important for successful murine colonization by *H. pylori* and FeoB mutants possess less cell-associated iron.[80] Additionally, *H. pylori* encodes a *fecAB* homolog, which may allow for the import of ferric dicitrate molecules, but homologs of the regulatory components found in *E. coli* are not present in *H. pylori*.[81] The FrpB outer membrane receptor is also present in *H. pylori*, which may allow for binding and transport of enterobactin during iron limitation.[82] In addition to these iron uptake systems, *H. pylori* contains the ferritin-like protein, Pfr, which helps prevent toxic iron accumulation by sequestering intracellular iron.[83]

Similar to Fur described in *Campylobacter*, *H. pylori* Fur exists in a holo-iron-bound form (holo-Fur) or in an unbound apo-form (apo-Fur) that imparts flexible functionality as a global regulator for gene expression in response to minor changes in iron availability.[84–86] *Helicobacter pylori* holo-Fur has been demonstrated to repress and activate gene transcription and apo-Fur has the capacity to regulate gene expression as well.[84–86] Upon encountering iron ions, Fur dimers bind to the target sequence within the promoter, effectively repressing transcription of the downstream genes. Many of these genes are involved in iron acquisition and include high-affinity iron transporters such as *fecA1*, *fecA2*, *frpB1*, *frpB2*, *frpB4*, *feoB*, *fecD*, *yaeE*, and the *exbB–exbD–tonB* operon.[82,87,88]

In addition to its role regulating central metabolism and iron acquisition, Fur also regulates a variety of virulence factors to promote pathogenesis. For example, holo-Fur has been shown to directly activate expression of genes involved in motility such as *fliY*, *flgK*, *flaB*, and *cheA*.[89] Additionally, Fur has been implicated in regulating the cytotoxin-associated gene, *cagA*, which encodes an oncogenic cytotoxin. Secretion of CagA by the *cag*-type IV secretion system (*cag*-T4SS) into host gastric cells results in a variety of changes in host cell signaling, morphology, and immunomodulation.[90,91] Translocation of CagA into host cells perturbs host cell polarity and apical release of transferrin, turning the cell surface into a replicative niche for *H. pylori*.[91,92] Interestingly, conditions of low iron availability induce production of *cag*-T4SS at the host–pathogen interface and enhance the activity of the *cag*-T4SS.[93–95] The importance of iron and Fur *in vivo* has been studied using the Mongolian gerbil model of chronic *H. pylori* infection. Results indicate that Fur is required for full colonization of the gerbil stomach at early points in infection.[96] Furthermore, interrogation of the Mongolian gerbil model of infection under conditions of varying iron concentration intake reveal that low dietary iron consumption exacerbates *H. pylori*-dependent carcinogenesis, a result that was not seen in iron-replete animals.[93]

Zinc

Zinc is an essential trace element necessary for various cellular functions in bacteria, including the proper structure and functioning of multiple metalloproteins involved in DNA replication, transcription, and other enzymatic processes.[97–100] To maintain appropriate levels of intracellular zinc, many bacteria employ both zinc uptake and efflux systems that aid in the ability of the organism to acquire exogenous zinc from the environment while preventing the accumulation of zinc to toxic levels.[101] For bacterial pathogens, zinc acquisition is often necessary to compete against residents of the host microbiota and allow for colonization of host tissues during infection.[54,98,101,102]

Susceptible hosts can also use zinc toxicity as a weapon against invading pathogens by preventing the bacterial cell from taking up other necessary metals during pathogenesis.[103] As a result, high levels of intracellular zinc must be avoided to prevent zinc outcompeting other necessary metals within the active sites of metalloproteins.[101]

Vertebrate hosts use both intracellular and extracellular strategies to limit access to zinc by invading pathogens.[104] Intracellular examples include the use of transporters to move zinc from the lysosome into the cytoplasm,[105] as well as intracellular sequestration of zinc by metallothionein,[106] to inhibit zinc-dependent bacterial processes. Hosts also use extracellular strategies, like S100 proteins, to sequester zinc and other metals making them unavailable to bacterial pathogens during infection.[101,105,107] These molecules can also serve as immune signaling molecules for the recruitment of neutrophils and monocytes during gastrointestinal infection.[104] In humans, the majority of zinc absorption occurs in the small intestines, severely limiting zinc availability to gastrointestinal pathogens like *Campylobacter*.[101] To combat host defenses and maintain zinc levels, both *Campylobacter* and *Helicobacter* have evolved several metalloregulatory mechanisms (Tables 1 and 2).

Campylobacter

To survive and proliferate in the zinc-limited environments within the host, *Campylobacter* uses efficient zinc uptake systems, including the ZnuABC transporter.[98,101,105,108] This system belongs to the ATP-binding cassette (ABC) family of transporters, with ZnuA serving as a periplasmic metallochaperone, ZnuB as the membrane permease, and ZnuC as the ATPase subunit.[101,109,110] ZnuA from *C. jejuni* has been shown to bind an average of 3.56 atoms of zinc per molecule and was required for successful colonization of chickens by *C. jejuni*.[101] Interestingly, it was subsequently demonstrated that ZnuA is not required for colonization of germ-free chicks, indicating the primary role of this system in chickens is to compete with the intestinal microbiota for zinc.[98] ZnuA was also found to be important for growth when competing for zinc in the presence of host-derived sequestration proteins such as S100A12.[104] As with other metals, an overabundance of zinc is toxic to *Campylobacter*, so the organism uses the conserved ZntA efflux protein to export excess zinc and other metals, keeping concentrations within the tight intracellular range necessary for survival.[111]

To maintain appropriate levels of zinc, the bacterium must also have regulatory mechanisms to detect and control expression of these uptake systems. *Escherichia coli* and other bacteria encode the zinc uptake regulator (Zur) that belongs to the Fur family of metalloregulatory proteins, to repress *znuABC* transcription. Though *znuABC* in *C. jejuni* was found to be upregulated in low-zinc environments,[101,105] BLAST analysis indicates that *Campylobacter* lacks a Zur homolog. Due to this absence, it has been suggested that *znuABC* is cross-regulated by PerR, Fur, or another unidentified regulator.[101] Support for PerR regulating *znuABC* comes from crystallization studies of *C. jejuni* PerR, where zinc and manganese binding were observed near the PerR active site, but direct interaction of PerR with the *znuABC* promoter has not been investigated.[112] In addition, *Campylobacter* encodes a putative cobalt–zinc–cadmium efflux protein, CzcD, which has been found to be important for *Campylobacter* colonization of the murine gut,[55] although regulation of these systems beyond response to presence of metals is currently unknown.

Helicobacter

Helicobacter pylori has a strict nutritional requirement for zinc in order to grow and proliferate.[77] During infection, *H. pylori* encounters decreased zinc concentrations within the inflamed gastric mucosa as a result of sequestration by host-derived S100A family proteins.[113–116] Conversely, humans consume large quantities of transition metals such as zinc in their diet, so it is likely that *H. pylori* is exposed to micromolar concentrations of this ion in the lumen of the stomach.[117] To persist in the gastric environment, *H. pylori* has evolved molecular mechanisms to sense and adapt to these wide variations in zinc concentration and availability.

While no zinc-specific import systems have been identified, several efflux systems have been studied in *H. pylori*. A homolog of the CzcABC efflux pump is encoded by *H. pylori*, although CzcC is absent and two copies of CzcB are present in the genome,[118] aiding in the prevention of toxic levels by exporting excess zinc. Another similar efflux system for zinc detoxification is CadA, which also prevents zinc accumulation by efflux of cobalt, cadmium, and zinc.[118] Similar to these two export systems, *cznABC* in *H. pylori* encodes a third efflux system to export zinc.[117] This transporter effluxes excess cadmium, zinc, and nickel as necessary, and is required for successful colonization of a host as demonstrated in the Mongolian gerbil model.[117] These systems function together to prevent toxic effects of metal accumulation, but the regulatory mechanisms of each beyond metal binding have not been studied.

Zinc regulates *H. pylori* virulence in several ways.[113,114,119] Conditions where zinc availability is low, such as those imposed by the presence of calgranulin proteins including S100A12, lead to repression of activity and assembly of major virulence factors such as the *cag*-T4SS.[113,114] Additionally, conditions of low zinc availability imposed by calgranulin proteins (such as calprotectin) also induce lipid modification, biofilm formation, and increased resistance to calgranulin antimicrobial activity.[119]

Although the molecular mechanism of these virulence properties remains unknown, *H. pylori* has evolved several ways to respond to varying conditions of zinc availability. Chelation of zinc can result in disruption of the Fur dimeric structure into monomers without associated zinc, impacting the ability of Fur to act in a regulatory manner.[120,121] Iron starvation, as well as medium supplementation with zinc, results in repressed synthesis of ferritin (at the mRNA level) in the wild-type strain but not in the *H. pylori fur* mutant, indicating zinc-associated *fur*-dependent regulation of *H. pylori* metal homeostasis occurs, and that Fur is a global metal-dependent regulator.[83]

Copper

The majority of environmental copper (80%) is found in shale and mining sites, with humans consuming an average of 2–4 mg of copper per day through diet and copper plumbing.[122] Copper is important to bacterial growth and pathogens will use host copper as a metal cofactor for enzymes involved in iron uptake and maintenance of the cellular oxidative state.[123,124] In addition to the use of copper in metalloenzymes, high levels of copper have been used for biocontrol due to its toxicity.[125] This toxicity may be the result of Fenton-like reactions that produce hydroxyl radicals[126] or the displacement of other metals, such as iron–sulfur complexes by copper ions.[127] As a result, not only is copper sequestered by the host to limit colonization, it may also be used in excess to combat bacterial pathogens.[128] Copper in a human host is primarily localized to the brain, kidneys, and liver, and is quickly sequestered by ceruloplasmin to maintain homeostatic

levels before excretion in bile.[122,128] Innate immune cells, such as macrophages, load their phagosomes with up to millimolar concentrations of copper to intoxicate bacteria.[129–131] The host also deploys copper-binding innate immune proteins such as S100A12, which can bind and sequester copper as an antimicrobial strategy.[114] As a result of copper requirements for growth, but the potential for toxicity, the ability to tightly regulate levels of copper is a requirement of bacterial pathogens, including *Campylobacter* and *Helicobacter* (Tables 1 and 2).

Campylobacter

Copper may serve to facilitate iron uptake and sense osmotic stress in *Campylobacter*[123] by acting as an iron-binding protein cofactor, increasing oxygen uptake via oxidation of periplasmic copper, and efflux of copper out of the cell under iron limitation.[66,123,124] However, copper can quickly become toxic at higher concentrations as previously described. Animal studies have demonstrated that supplementation of pig feed with exogenous copper sulfate and carbadox decreased shedding of *Campylobacter*[132] and biofilm formation decreased on a surface containing copper with an ~2-fold decrease in viable cells (CFU/ml).[133] These results indicate that increased levels of exogenous copper can be detrimental to *Campylobacter* growth and that there is potential therapeutic value for copper combating *Campylobacter* infection. As such, regulating cellular copper levels is likely important to *Campylobacter* physiology.

The periplasmic protein P19 (mentioned above) also aids in the ability of *Campylobacter* to use copper and prevent toxicity.[66,134] Similar to iron chelation studies conducted with wild-type and P19 mutants, chelation of copper with bathocuproine disulfonic acid under iron-replete conditions resulted in decreased growth of both wild-type and mutant strains,[66] indicating a potential role of copper in iron uptake by *Campylobacter*. In addition, copper also regulates intracellular oxygen via the cytochrome *c* oxidase complex.[123] A homolog of *E. coli* CueO, the multicopper oxidase in *C. jejuni* exhibits similar activity to CueO by coupling oxidation of copper-containing substrates to the reduction of oxygen to water.[123,124] *Campylobacter jejuni* CueO acts in conjunction with a CopA homolog to remove and detoxify copper from Cu^{+} in the cytoplasm to Cu^{2+} in the periplasm.[123,124,135,136] While regulation of these genes has not been investigated in depth, the twin arginine translocase TatA1 was shown to be required for correct periplasmic localization of *Campylobacter* CueO[137] and the *Campylobacter* oxidative stress regulator (CosR) has been implicated as a negative regulator of *cueO* in a CosR knockdown study[138]; however, much is still unknown regarding the exact mechanisms regulating these systems.

Helicobacter

Helicobacter pylori encounters micromolar concentrations of copper in the gastric niche, largely derived from the host diet and present at the lumen of the stomach.[139] Copper has been shown to influence bacterial cellular movement and acts as a chemotactic repellent for *H. pylori* motility.[140,141] Copper also promotes *H. pylori* colonization of the rodent stomach, indicating this transition metal is important at the host–pathogen interface.[140] Additionally, copper is required for cellular respiration and is a critical cofactor in the terminal oxidase in the *H. pylori* respiratory chain, a cbb3-type cytochrome oxidase that possesses a heme–copper binuclear center similar to the cytochrome aa3-type oxidases.[142]

Copper induces the expression of numerous *H. pylori* genes involved in ion transport, including *exbD*, *crcB*, *HP1516*, *HP0733*, and the *crdAB*, *czcAB*, and *copAP* operons.[118] The *exbD* gene encodes the energetic component of the iron and nickel transport system (ExbB–ExbD–TonB). *Helicobacter pylori* employs a two-component system *crdRS* that encodes a sensor kinase (CrdS) and a response regulator (CrdR) that comprise a phosphorelay that is activated in the presence of copper.[143] CrdR induces the expression of *crdAB* and *czcAB* operons that encode copper resistance determinants such as secreted copper-binding molecule CrdA, and a copper efflux pump comprised of CrdB, CzcB, and CzcA (Fig. 3). Because evading copper intoxication is clearly a critical strategy for *H. pylori*, it also has functional redundancy with numerous copper export systems, such as the *copAP* operon, which encodes a cytoplasmic copper-binding regulator (CopP) that is orthologous to CopZ from *E. coli*. CopP promotes expression of *copA*, which encodes a protein that promotes *H. pylori* resistance to copper.[144] Copper exposure alters expression of genes implicated in several metabolic pathways, such as inducing expression of *trpA* that encodes tryptophan synthase and *hpylM* that encodes a DNA methyltransferase.[118]

Helicobacter pylori has evolved elegant mechanisms to sense and respond to copper levels and deploy copper detoxification strategies.[117,143] *Helicobacter pylori* alters its transcriptional profile in response to copper exposure by reducing the expression of the *pfr* gene that encodes the metal storage protein, ferritin. Interestingly, this copper-induced downregulation of ferritin is Fur dependent, indicating Fur is a global metal stress regulator in *H. pylori*.[83] In addition to ferritin, numerous additional genes are regulated by copper exposure, including those involved in ion transport, cellular motility, transferase activity, and metabolic flexibility. Copper exposure also results in the upregulation of *fliS* that encodes a putative flagellin chaperone.[118] Additionally, these transcriptional changes may be the indirect result of copper displacing iron ions, as observed in *E. coli*.[127] Thus, copper can influence *H. pylori* motility at both the chemotactic and transcriptional levels.

Nickel

Nickel is widespread throughout the natural environment at very low levels.[145] Small amounts of nickel (3–4 nM) are required for cellular processes in bacteria and humans, but due to the ability of nickel to displace other metal cofactors, much research has focused on the toxic effects of nickel.[145–148] The majority of host nickel is located in organs, such as the lungs and adrenal glands, at incredibly low concentrations (<5 ppm).[147,149,150] These low levels are maintained in part because dietary nickel is either eliminated in the feces or excreted in the urine.[147,150] The host can also utilize metal sequestering molecules, such as S100A8/A9, to bind residual nickel and aid in further limitation of available nickel to invading bacterial pathogens.[149,151] Due to both the necessity of nickel and its potential toxicity, sensitive transport and regulatory systems are vital to bacterial survival during infection (Tables 1 and 2).

Campylobacter

Nickel is essential for various cellular functions in *Campylobacter*. For example, nickel is involved in hydrogenase activity,[152] with *C. jejuni* [Ni–Fe]-hydrogenase catalyzing the reversible oxidation of H_2.[153] It was previously thought that *Campylobacter* did not possess a nickel transporter like the *nikABCDE* transport system in *E. coli*, and instead used enzymes dependent on nickel cofactors,[154,155] but the *nikZYXWV* (Cj1584c–Cj1580c) gene

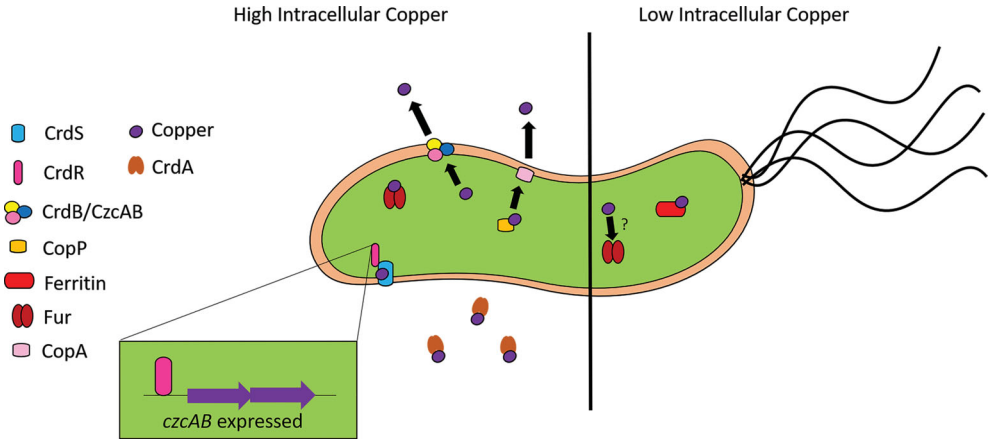

Fig. 3 Metalloregulatory mechanisms expressed during high and low intracellular copper concentrations in *Helicobacter*. High levels of intracellular copper induce activity of the response regulators CrdR, CopP, and Fur. The induction of *czcAB* expression by CrdR is shown as an example of transcriptional changes in response to copper. Increased levels of copper induce the expression of the CrdB and CzcAB efflux system, as well as the CopA efflux system, and the secretion of the copper-binding protein CrdA to help maintain homeostatic levels of copper. In low levels of intracellular copper, ferritin binds available copper, in addition to other metals, but this sequestration is reversed when high levels of copper are available.

cluster encoding an outer membrane transporter has since been described in *C. jejuni*.[152] The periplasmic binding protein component of this system (NikZ) is similar to NikA of *E. coli*,[152,156,157] but they exhibit drastically different binding specificities. While purified *Campylobacter* NikZ was shown to only bind free or uncomplexed nickel, NikA can bind complexed forms including Ni chelated with EDTA.[152,158–160] Once inside the periplasm, nickel is bound by the HydABCD complex (Cj1267–Cj1264 in 11168) resulting in the oxidation of hydrogen to release protons and facilitate the proton-motive redox loop with the uptake of protons during reduction of menaquinone.[152,160]

Since HydABCD activity is dependent on nickel availability in the periplasm,[155] its function is impacted by the abundance of the Nik protein. For example, NikZ can be detected in the periplasm of *C. jejuni* cells under normal conditions, but following supplementation with exogenous nickel, NikZ was unable to be detected in the periplasm, suggesting that *nikZYXWV* expression is controlled by nickel availability.[152] Most strikingly, when grown under the low-nickel conditions like those *Campylobacter* may encounter in a host, mutation of NikZ resulted in an ~22-fold decrease in cell-associated nickel and hydrogenase activity was lost. Despite this mutation, hydrogenase activity could be restored when supplemented with exogenous nickel indicating that additional nickel transporters may exist.[152]

Regulation of these systems beyond the impacts of nickel concentrations either is poorly understood or has not been investigated. Several nickel-responsive regulators such as the Fur-like transcription factor, Nur, and the *nik* gene regulator, NikR, have been identified in other Gram-negative organisms, but homologs of these regulators either do not exist or are not widely distributed throughout the *Campylobacter* genus and have regulatory abilities that have not been investigated.[152,161] In the 13826 strain of *Campylobacter concisus*, a putative NikR (13826_0355) was identified based on genome sequence, but the ability of this proposed NikR homolog to regulate the NikZYXWV system has not been investigated.[162]

Helicobacter

Similar to *Campylobacter*, *H. pylori* requires nickel for certain cellular processes like virulence, but also can be intoxicated by this transition metal.[163] Two critical *H. pylori* enzymes require nickel as a cofactor: urease and [Ni–Fe]-hydrogenase. Both urease and [Ni–Fe]-hydrogenase require the protein HypB for the metalation-dependent maturation of these enzymes.[164] Urease is an essential colonization factor that facilitates *H. pylori* survival in the acidic conditions encountered in the lumen of the gastric mucosa.[163] *Helicobacter pylori* urease is a nickel-containing enzyme that catalyzes the hydrolysis of urea to ammonium carbonate.[165–168] *Helicobacter pylori* requires nickel for the full maturation of the urease complex.[169–172] Specifically, expression of urease genes is increased in response to nickel exposure. This likely evolved to repress expression when the critical cofactor for this enzyme is low.[173]

In conditions of nickel stress or toxicity, *H. pylori* expresses the Hpn and HspA proteins that participate in metal storage and protect the cells from higher concentrations of external metal concentrations, including nickel.[174] Together, these residues bind metal ions with high affinity, to sequester them within the cytoplasm of the cell and overcome the challenge of metal toxicity. Nickel exposure also induces expression of the *cznABC* loci that encode components of a putative nickel efflux pump.[117] This CznABC transport system is critical for circumnavigating nickel stress and is required for colonization of the vertebrate host, indicating nickel toxicity is a challenge in the gastric niche.[117]

In addition to Fur-dependent regulation of urease gene expression in response to nickel, the dominant transcriptional regulator associated with nickel response is NikR. NikR can bind nickel or copper with picomolar affinity, in what is likely a conserved square-planar site.[175] NikR is autoregulatory, binding to the promoter sequence upstream of the gene that encodes it in the presence of nickel.[175,176] Comprehensive mapping of the NikR regulon by RNA-seq and ChIP-seq indicates 194 differentially expressed genes between the wild-type and *nikR* mutant strains, but <50 differentially regulated in response to nickel between these two strains (20 were identified by RNA-seq and 46 by ChIP-seq), indicating NikR regulates a variety of genes in a nickel-independent fashion.[177]

Holo-NikR (the nickel-bound form of NikR) represses HcpC, HopV, and HopW, which are all outer membrane or secreted proteins involved in host–pathogen interactions.[178,179] Additionally, holo-NikR represses expression of the DvnA-RS03305 and

MccABC toxin–antitoxin systems, Fur, and the ScoB and AtoE proteins involved in metabolic processes.[179] Interestingly, holo-NikR induces expression of urease subunits, which utilize nickel as a cofactor for activity. In addition to urease, the other critical enzyme regulated by holo-NikR is the [Ni–Fe]-hydrogenase. [Ni–Fe]-hydrogenase is a nickel-containing enzyme that enables *H. pylori* to utilize hydrogen gas within the gastric niche as an energy source.[164] NikR has also been implicated in regulating biofilm formation by *H. pylori* as mutational inactivation of *nikR*, *fur*, and the acid-responsive two-component regulatory system encoding ArsRS in double and triple mutants enhances biofilm formation.[180] Interestingly, NikR has been implicated as a critical regulator of virulence and isogenic *nikR* mutants exhibit a colonization defect in rodent models of *H. pylori* infection.[181]

Other genes that are regulated by NikR in response to nickel stimulation include FecDE, CeuE, FrpB2, FrpB3, and FecA3, which are involved in metal ion transport and are repressed by holo-NikR.[177] It is interesting to note that the FrpB4/FrpB2, FecDE ABC transporter, CeuE transporter, and a TonB1–ExbB–ExbD system contribute to nickel acquisition as well as iron acquisition, underscoring the promiscuity of these critical transporters.[182] Furthermore, NixA, a nickel permease required for nickel transport from the periplasm into the cytoplasm, is repressed by holo-NikR.[177] Recently, a novel *H. pylori* nickel transport system, *NiuBDE*, was discovered that participates in nickel acquisition and nickel-dependent urease activation and is essential for colonization of the mouse stomach.[183]

Cadmium

Cadmium is a non-essential trace metal that can occur naturally,[184,185] although industrial cadmium is ubiquitous in the environment due to industrial contamination.[186] At elevated concentrations, cadmium can be toxic due to its ability to replace other divalent cationic metals, such as zinc and calcium.[186,187] In both animals and humans, a role for cadmium has not been identified and cadmium has been shown to induce cancer.[188] As a result, it is necessary for both hosts and pathogens to tightly regulate the amount of cadmium to prevent toxicity (Tables 1 and 2).

Campylobacter

While it does not appear that *Campylobacter* uses cadmium for any biological process, cadmium has been shown to reduce *Campylobacter* colonization of the murine gut[189] and cadmium chloride susceptibility was proposed as a means to differentiate *Campylobacter* spp. from other enteric pathogens.[189] Levels as low as 0.1 mM cadmium chloride significantly decreased growth of *C. jejuni*, with concentrations of 1 mM required to eliminate viability.[190] Supplementation of media with either blood or fluid thioglycolate rescued cells from these toxic effects.[191] The basis for cadmium toxicity in *Campylobacter* is not well understood, but several mechanisms for decreased cadmium toxicity have been proposed, including increased intracellular Zn^{2+} concentrations, decreased Cd^{2+} uptake, increased expression of metallothionein (a protein that sequesters cadmium), and binding of cadmium ions by other metal sequestering proteins.[190,192,193] A change in extracellular cadmium concentrations on a micromolar scale is enough to elicit a bacterial response, where transcripts involved in metal uptake such as bacterioferritin (*cj1534c*) and an ATP-binding protein (*cj1663*) were downregulated in the presence of cadmium, while those involved in metal storage like ferritin (*cj0612c*) were upregulated.[190] The CzcABCD efflux system may also help promote cadmium resistance in *Campylobacter*, similar to the role of cadmium efflux pumps identified in other organisms.[194,195] However, the mechanisms regulating these systems are currently unknown.

Helicobacter

Cadmium is not nutritionally required for *H. pylori* growth,[77] but similar to *C. jejuni*, *H. pylori* is quite sensitive to cadmium (Cd^{2+}) toxicity. *Helicobacter pylori* mitigates cadmium stress by elaborating a putative transition metal ATPase, CadA,[196,197] and a metal efflux pump, CznABC,[117] which aid in resistance to Cd^{2+}. Additionally, Hpn and HspA proteins from *H. pylori* participate in metal storage and protect the cells from higher concentrations of external metal ions like cadmium.[174]

Cobalt, magnesium, and manganese

Campylobacter

Cobalt is a required component of the B12 coenzyme complex, which plays a role in methyl group transfer and can interact with RNA regulatory elements to impact gene expression.[154,198] There is little known regarding the import of cobalt in *Campylobacter*, but a putative cobalt–zinc–cadmium efflux system, CzcD,[55] and a cytoplasmic transport protein, CorA,[152,199,200] with the potential to bind cobalt have been identified in the *C. jejuni* genome. Cobalt has been investigated as an antimicrobial coating with visible light-activated cobalt nanoparticles exhibiting bactericidal activity against *C. jejuni* and other Gram-negative pathogens, which opens the door for its use as a future antimicrobial surface treatment.[201]

Magnesium is necessary for colonization of the host gut by *Campylobacter*, but the majority of magnesium within the host is either sequestered in bone or complexed with nucleic acids.[202] Therefore, *Campylobacter* has developed methods to acquire non-sequestered magnesium from the host.[203] CorA is the primary transporter of magnesium in *C. jejuni*, which is a homolog of CorA transporters in other bacteria, including *E. coli*.[203] While little is known about the mechanism of magnesium transport, CorA mutants have been shown to require exogenous magnesium when grown under magnesium-deplete conditions, but these mutants have not been studied *in vivo*.[203] Additionally, CorA is thought to transport iron by acting as a low-affinity transporter under conditions of low magnesium.[204] Despite the requirement of magnesium for growth, the introduction of elevated levels of exogenous magnesium oxide to the environment is detrimental to *Campylobacter* more than most other bacteria since it is particularly sensitive to membrane damage.[205]

Manganese is a transition metal used by *Campylobacter* during colonization for the proper functioning of multiple proteins, including pyruvate kinases and catalases,[105] and is also predicted to be necessary for PerR activity during periods of iron limitation.[76] In humans, the majority of manganese is acquired through the diet and is present in the gastrointestinal tract.[206,207] Because of the importance of this metal for both host and bacterial cellular functions, humans employ several methods of manganese limitation, including sequestration by S100A8/A9[208] and removal of manganese from the phagosome by natural resistance-associated macrophage protein 1.[207,209] To compete with these host mechanisms, *Campylobacter* uses the MntABC transporter, which is similar to the ZnuABC zinc transporter described previously.[105] A regulatory role for PerR in MntABC expression has been identified in *Neisseria gonorrhoeae*,[210] but the role of PerR in *mntABC*

regulation in *Campylobacter* has not been investigated. Beyond MntABC, other manganese-specific transporters have yet to be identified, although other metal transport systems and binding proteins can interact with manganese nonspecifically.[105,200,207,211]

Helicobacter

Cobalt is a micronutrient that is nonessential for *H. pylori* growth,[77] but is required for the activity of important enzymes such as arginase. Arginase is a metalloenzyme that hydrolyzes arginine to ornithine and urea. Most arginases are binuclear and utilize Mn^{2+} as a cofactor; however, the *H. pylori* arginase enzyme is selective for cobalt.[212] Cobalt acquisition is mediated in *Helicobacter* spp. by the FecDE ABC transporter, CeuE transporter, and a TonB1 ortholog.[182] NiuBDE also transports cobalt into the bacterial cell[183] and NixA has been implicated in cobalt uptake.[213] Additionally, cobalt competes with nickel in metal acquisition pathways indicating cobalt and nickel transporters are promiscuous for these two transition metals.[214] In addition to being an important cofactor in enzymes, cobalt can be bactericidal against *H. pylori*.[214] In response to cobalt exposure, *H. pylori* deploys efflux determinants such as CadD. Plasmid-based expression of *cadA* in wild-type or *cadA* mutant strains of *H. pylori* enhanced resistance to cobalt (Co(II)).[196]

Magnesium is a strict nutritional requirement for *H. pylori* growth and viability[77] and is essential for *H. pylori* colonization in the porcine model of colonization and disease.[215] Interestingly, systemic magnesium levels are significantly lower in *H. pylori*-infected individuals compared to uninfected individuals.[216] The uptake of magnesium in *H. pylori* is mediated by the transporter CorA,[217] which is essential for the viability of *H. pylori*. Manganese is another transition metal that is dispensable for *H. pylori* growth,[77] but can be toxic to *H. pylori* cells. Interestingly, inactivation of the gene *pfr* that encodes *H. pylori* ferritin results in enhanced sensitivity to manganese toxicity, indicating metal storage is critical for *H. pylori* to overcome metal toxicity.[218]

Bismuth, molybdate/molybdenum, and tungstate/tungsten

Campylobacter

While bismuth has been used for many years to treat stomach ulcers by reducing *Helicobacter* colonization in humans, little is understood about the effects on *Campylobacter*.[219] However, bismuth has recently been shown to reduce the ability of *Campylobacter* to colonize the chicken gut.[219] Upon dosing chickens with either 0, 50, or 200 ppm, it was determined bismuth administered at 200 ppm for 10 days lowered concentrations of *C. jejuni* in the chicken gut,[219] but the specific mechanisms by which bismuth affects *Campylobacter* have yet to be determined.

Campylobacter is exceptional among other microorganisms in that it encodes cofactor proteins and transporters for both molybdenum and tungsten.[50] Molybdenum and tungsten are transition metals of similar atomic conformations and present in the environment in the usable forms of molybdate and tungstate,[220] which can be used interchangeably, though the relative abundance of molybdenum is higher in relation to tungsten.[221,222] In *Campylobacter*, molybdenum and tungsten are transported by ModABC and TupABC transporters, respectively.[50,223] Interestingly, the ModA transporter can transport either metal while TupA is believed to be tungsten-specific, since TupA-like transporters in other organisms exhibit high specificity into the picomolar range.[222] Expression of these transport systems in *Campylobacter* is controlled by a regulatory protein similar to ModE in other microbes.[50,223] Due to the lack of a specific metal-binding domain, it is thought that *Campylobacter* ModE regulates both metal uptake systems based on protein–protein interactions, rather than direct interaction of ModE and molybdate or tungstate.[50,224]

After transport, the respective metals are used by either the molybdenum cofactor (Moco), which is involved in nitrate reductase activity, or the tungsten cofactor (Woco), which acts as a cofactor for formate dehydrogenase during energy metabolism.[50] In addition, several other enzymatic cofactors utilize molybdenum, including the periplasmic nitrate reductase NapA.[222,225] While the use of tungsten is less understood, it has been shown to be required for proper functioning of formate dehydrogenase[50] and can inhibit some molybdenum enzymatic cofactors, though the mechanism remains to be elucidated.[222]

Helicobacter

Bismuth is considered a common first-line therapy for patients with penicillin allergies suffering from *H. pylori* infection.[226] Bismuth, in combination with other antimicrobial compounds such as rabeprazole, cefuroxime, levofloxacin, esomeprazole, or clarithromycin, is effective at eradicating *H. pylori* in chronically infected patients.[226,227] Standard triple therapy in combination with bismuth results in complete eradication of *H. pylori* in 90% of patients.[228] Bismuth is not required as a nutrient for *H. pylori* growth,[77] but can be toxic to *H. pylori* cells, underscoring its importance as an antimicrobial therapy. Bismuth can inactivate important transcriptional regulators required for *H. pylori* colonization and pathogenesis, such as NikR.[229]

Molybdenum is a rare element essential to nearly all organisms that catalyzes two-electron-transfer oxidation–reduction reactions. Growth analyses demonstrate that molybdenum is dispensable for *H. pylori* growth and viability.[77] However, some *H. pylori* strains encode a putative ABC transporter, ModD, involved in molybdenum transport from the periplasm into the cytosol.[230] Phylogenetic analyses of *H. pylori* strains from East Asian, Amerindian, European, and African individuals have revealed that all known functions associated with molybdenum appeared to be inactivated in East Asian strains, further underscoring the dispensability of this metal ion within the genus.

Tungsten is not required for *H. pylori* growth and viability.[77] However, tungsten in the form of polyoxotungstates exhibits potent antibacterial activity against *H. pylori*, an effect that was superior to metronidazole and clarithromycin, both standard therapies for this pathogen.[231] Additionally, polyoxotungstates were effective against metronidazole-resistant and clarithromycin-resistant strains of *H. pylori*, indicating the potential of these molecules as metal-based therapeutics.

Conclusions

Iron

The importance of iron acquisition for survival of bacterial pathogens like *Campylobacter* and *Helicobacter* is evidenced by the array of iron uptake systems present in these organisms. Ranging from free iron transport systems to those transporting iron-bound siderophores produced by other bacteria, pathogenic Epsilonproteobacteria can obtain iron from various sources and in numerous forms. The ability to use iron in various forms is reflective of the specific niches occupied by *Campylobacter* and *Helicobacter*. With the ability to survive in environmental niches ranging from

aquatic sources to the human gut, *Campylobacter* must employ numerous systems to obtain iron from a variety of potential sources. Similarly, *Helicobacter* uses multiple uptake systems, but within a very specific environment in the gastric niche. The induction of host responses, such as inflammation during infection, may generate a preference for certain iron acquisition systems over others, such as those that can bind and take up heme from blood released into the lumen during *Campylobacter* infection. Additionally, iron abundance in the environment and varying levels of oxidative stress encountered during colonization likely impact preferences for certain regulatory systems over others.

Understanding the molecular mechanisms and conditions impacting the regulation of these iron uptake systems can contribute significantly to our understanding of pathogenic Epsilonproteobacteria physiology. Previous studies have indicated a vital role for the global response regulators Fur and PerR, in the transcriptional response of *Campylobacter* and *Helicobacter* iron transport systems in response to environmental levels of iron. While these global regulators are important for regulation of virulence factors during infection, other regulators identified recently, such as HeuR in *Campylobacter*, are worth investigating further. The abundance of iron acquisition systems in these pathogens and the ability of some systems to bind and transport other metals indicate the potential for previously unidentified or uncharacterized regulatory mechanisms to work in conjunction with global regulators like Fur. By focusing on these potential regulatory systems beyond Fur and PerR, we can gain a better understanding of the complex interactions of iron acquisition systems and the ability of *Campylobacter* and *Helicobacter* to successfully colonize susceptible hosts.

Zinc

Competition for zinc within the host gut during infection is of interest, as the gut is a major site of zinc absorption in humans. Due to this absorption by the host and sequestration by the native microflora, pathogens must be able to compete with the host and the host microbiome to scavenge available zinc existing at a very low abundance. These environmental pressures encountered in the host have resulted in the ability of *Campylobacter* and *Helicobacter* to use efficient zinc acquisition and transport systems to help combat zinc sequestration by host-derived molecules such as S100A8/A9. In addition to zinc acquisition, investigation of these systems demonstrated how some may be able to transport iron or other essential metal nutrients as well, allowing the pathogens to circumvent host nutritional immunity based on metal availability.

Understanding how these pathogens can combat host defenses and sequester zinc is increasingly important due to the employment of zinc treatment as a nutritional supplement in the developing world. Supplementation of zinc in the diet of humans in developing countries may lead to an increase in available zinc levels in the gastric and intestinal niches, resulting in heightened host susceptibility to infection by *Helicobacter* or *Campylobacter*, respectively. While some zinc uptake systems have been characterized in *C. jejuni*, such as ZnuABC, the promiscuity of transport systems for multiple metals and limited known regulatory mechanisms have led to knowledge gaps in the field. *Helicobacter* differs from *Campylobacter* as no zinc importers have been identified, but several zinc efflux systems are important to combat potential zinc toxicity in the gastric niche. However, regulation of these systems is not understood beyond the global response to zinc availability. Because of this, it is worth determining whether zinc transport occurs in a hierarchical manner and what changes occur in the response of transport systems under differing conditions, and which systems are most important, in addition to gaining a better understanding of the regulatory mechanisms that control these systems. This would provide insight into potential methods for prevention and treatment, specifically within susceptible hosts residing in the developing world.

Copper

Unlike iron that requires high intracellular concentrations before reaching deleterious levels, copper can quickly become toxic due to the production of reactive radicals or protein mismetallation.[232] Prevention of copper accumulation may explain the pairing of iron- and copper-binding sites within a single transporter. This dual binding in certain pumps can facilitate the efflux of copper while maintaining homeostatic levels of iron; however, this may lead to problems if mutations occur within these efflux systems and intracellular copper accumulates. A better understanding of how these efflux pumps function in copper homeostasis and how copper competes with other nutrient metals for binding sites could lead to exploitation of these mechanisms to combat infection due to the toxic effects of copper accumulation.

Work investigating the global impact of copper on genes in *Helicobacter* has been done, but research into understanding the effects of copper on *Campylobacter* gene expression is lacking. The impact of copper on *Campylobacter* has not been a major focus of metalloregulatory work, but using available knowledge on copper toxicity and the impact on *Helicobacter* virulence to inform future research can lead to a better understanding of how *Campylobacter* regulates copper levels via finely tuned transport mechanisms. We can also gain a better understanding of the interplay between copper and other metals and how the availability of these metals during colonization can impact transcription of virulence genes within invading pathogens.

Nickel

Although nickel is necessary for various cellular functions within *Campylobacter* and *Helicobacter*, little is known regarding regulation of nickel transport and utilization by these organisms when compared to the dearth of knowledge available for other metals like iron. While it has been demonstrated that *Campylobacter* requires nickel, a nickel-specific transport system similar to the NikABCDE system in *E. coli* and *H. pylori* was only recently described in *Campylobacter.* While nickel-specific transporters have been identified for both organisms, the promiscuity of nickel binding is not well understood beyond studies indirectly confirming nickel may bind to other metal transporters. Additionally, the toxic potential of nickel based on the ability of nickel to bind to other enzymatic metal-binding sites is worth noting. As seen in other mechanisms involving transition metals such as zinc, metal regulatory systems in Epsilonproteobacteria can often bind more than one type of metal. This lack of specificity may be important to combat host nutritional immunity and compete with the native microflora, so investigating these identified mechanisms further and characterizing other proposed systems would provide useful insights into the ability of pathogens to regulate metal transport through potentially interconnected systems. The impact of nickel on other metal transport systems is worth investigating as novel systems that have yet to be identified may exist in these organisms, allowing for the ability to successfully evade host nutritional immunity in previously uncharacterized ways.

Cadmium

Cadmium is unique among the transition metals described in this review because there is no biological requirement for intracellular cadmium in Epsilonproteobacteria and toxic effects are often experienced by cells due to cadmium accumulation. As a result, these organisms have evolved several methods for dealing with exogenous sources of cadmium, although these processes have not been studied in depth and understanding of the regulatory mechanisms is severely lacking. It is worth noting the impact of cadmium on the regulation of proteins related to the uptake and storage of other metals such as iron and zinc. This sequestration of other metals to avoid uptake of cadmium would be an interesting route to investigate as the accumulation of these other metals can be toxic to the cell. The ability to regulate intracellular levels of certain metals to avoid the uptake of others like cadmium showcases the intertwined metalloregulatory mechanisms of Epsilonproteobacteria and lends support to hypotheses that these regulatory systems work in tandem to maintain homeostasis, especially during infection.

Cobalt, magnesium, and manganese

Cobalt, magnesium, and manganese are among the less well studied enzymatic cofactors in Epsilonproteobacteria, but they encode transporters for either uptake or efflux of each metal to maintain a healthy equilibrium. Although cobalt can serve as a cofactor for some enzymes, the ability to combat cobalt toxicity is necessary to survive in the host environment. Additional metals like magnesium and manganese have not been investigated as thoroughly as iron and zinc, but Epsilonproteobacteria seem to diverge in the ability to tolerate these metals. While *Campylobacter* requires both to serve as cofactors for different metalloenzymes and utilizes transporters to acquire these metals, *Helicobacter* does not require manganese for growth and can only tolerate very low levels of the metal. Further research is required to tease apart the mechanisms, but the ability to utilize magnesium and manganese may be a distinguishing characteristic between members of the Epsilonproteobacteria. Beyond the transport systems associated with these metals, our understanding of the regulatory mechanisms impacting these systems is sorely lacking and could be the focus of future metalloregulatory work in Epsilonproteobacteria.

Bismuth, molybdate/molybdenum, and tungstate/tungsten

Unlike the more common transition metals discussed in this review, very little is understood about the biological role of bismuth in Epsilonproteobacteria. Bismuth has been used historically to treat *Helicobacter* infections, but the mechanistic impact of this metal is poorly understood. *Campylobacter* is also negatively impacted by bismuth, but the molecular mechanisms behind this detrimental effect are not known though it could be a potential therapeutic agent. Interestingly, the reverse is true for molybdate/molybdenum and tungstate/tungsten in Epsilonproteobacteria, especially *Campylobacter.* Transporters with high specificity for both metals have been identified in *Campylobacter*, while we only have a general understanding of those in *Helicobacter*. The specificity of these transporters in *Campylobacter* seems unique among Gram-negative organisms, so the characterization of these systems may provide insights into new systems in other pathogenic organisms. Although the identity of these metal transporters is known and a general regulatory mechanism has been identified, understanding of the exact roles of molybdenum and tungsten within Epsilonproteobacteria is lacking.

During the infection state, hosts sequester transition metals as a means of nutritional immunity. To combat the host defenses and obtain nutrient metals, pathogenic Epsilonproteobacteria must employ different mechanisms to transport metals and maintain homeostasis. Taken together, these conclusions demonstrate that although numerous regulatory systems have been identified in Epsilonproteobacteria, many gaps still exist in our understanding of the global impact of metals on the physiology of *Campylobacter* and *Helicobacter.* Additionally, our understanding of how these organisms are able to finely regulate these systems to avoid metal toxicity is limited and worth further investigation. The focus of this review was at the host–pathogen interface, but the role of the native microbiome may also have an impact on the ability of these pathogens to acquire and transport metals, and should be taken into account in future studies. The potential for additional metal transport systems that remain to be characterized and the divergence of Epsilonproteobacteria from other more well-studied organisms, such as *E. coli*, lend support to further investigate the systems in these organisms since we will likely gain novel insights into strategies used by pathogenic bacteria to acquire and regulate metals.

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Author contributions

BRK, JL, KPH, JAG, and JGJ conceived, designed, wrote, and edited this manuscript for critical content. All authors approved this manuscript for content integrity and accuracy.

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Conflicts of interest

There are no conflicts of interest to declare.

Data availability

No data was produced for this review.

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