Abundant Monovalent Ions as Environmental Signposts for Pathogens during Host Colonization

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ABSTRACT Host colonization by a pathogen requires proper sensing and response to local environmental cues, to ensure adaptation and continued survival within the host. The ionic milieu represents a critical potential source of environmental cues, and indeed, there has been extensive study of the interplay between host and pathogen in the context of metals such as iron, zinc, and manganese, vital ions that are actively sequestered by the host. The inherent non-uniformity of the ionic milieu also extends, however, to “abundant” ions such as chloride and potassium, whose concentrations vary greatly between tissue and cellular locations, and with the immune response. Despite this, the concept of abundant ions as environmental cues and key players in host-pathogen interactions is only just emerging. Focusing on chloride and potassium, this review brings together studies across multiple bacterial and parasitic species that have begun to define both how these abundant ions are exploited as cues during host infection, and how they can be actively manipulated by pathogens during host colonization. The close links between ion homeostasis and sensing/response to different ionic signals, and the importance of studying pathogen response to cues in combination, are also discussed, while considering the fundamental insight still to be uncovered from further studies in this nascent area of inquiry.

KEYWORDS abundant ions, ClC chloride channels, *Mycobacterium tuberculosis*, chloride, host colonization, host-pathogen interactions, ionic homeostasis, potassium

Ions are a fundamental basis of life, essential in the biology of all organisms, with roles in signaling, as cofactors in vital enzymes, and in secondary transport of core nutrients (1–7). In the context of infection, ions that are scarce within the host (via sequestration or as trace metals) but required by the pathogen have been the topic of intense study (5, 8, 9). This is exemplified by work on ions such as iron, zinc, and manganese, with numerous studies providing insight into the biological functions and acquisition/sequestration methods on the sides of both the host and the pathogen (5, 8, 9). In contrast, the role that abundant ions such as chloride (Cl[−]), potassium (K⁺), and sodium (Na⁺) may play in infection has been much less considered and appreciated. Unlike the former class of ions, which are actively sequestered by the host and that often play roles in nutritional immunity, these abundant ions cannot be sequestered and are present in high-millimolar quantities in specific sublocations within the host. While they are a ubiquitous presence in experimental assays as counterions, their contributing role in infection biology has largely been overlooked. Critically however, the concentrations of these “abundant” ions are not uniform throughout the host, but rather differ in defined manners depending on location and immune response (see for example references 10–13), marking them as potentially ideal environmental cues for pathogen exploitation during host adaptation and colonization.

This review focuses in particular on Cl[−] and K⁺, highlighting their roles in host cell/organismal biology and drawing together studies of multiple bacterial and parasitic species that have collectively begun to show: (i) how the defined concentration changes of

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these abundant ions by location and immune response are exploited as environmental cues by pathogens during infection, (ii) the active microbial modulation of host levels of these ions that can occur, and (iii) the close links between homeostasis and sensing/response to disparate ions.

CHLORIDE

Chloride is the most abundant anion in biological systems. Human plasma contains 94 to 111 mM chloride (Cl^-) (14), with intracellular levels of ~ 30 to 50 mM but with further variation depending on factors such as cell type and developmental stage (15–18). Cl^- plays multiple fundamental roles in host cell/organismal biology, including as a counterion in ensuring electroneutrality, in modulating excitability of cells such as neurons and muscle cells, in driving water movement and thus cell volume regulation, and in activating enzymes such as cathepsin C and WNK (with no lysine [K]) kinases (7, 19, 20). There is an extensive suite of Cl^- channels and transporters that work in mediating Cl^- movement across the plasma membrane or across vesicular compartments, some of which function as Cl^- /proton (H^+) exchangers, and their importance in a diversity of functions is underscored by the breadth of diseases associated with loss of function of these various systems (19). These range from myotonia, where skeletal muscle is impaired in relaxation (CIC-1) (21–23), to cystic fibrosis, where disruption of Cl^- movement affects water movement and mucus secretion (cystic fibrosis transmembrane conductance regulator [CFTR]) (24, 25). It further encompasses several diseases arising from improper acidification or ion homeostasis within endosomal compartments, such as renal Dent's disease (CIC-5) (26–29), osteopetrosis (CIC-7) (30, 31), and neurodegeneration (e.g., CIC-3 and CIC-7) (32–35).

In the context of infection, the role of Cl^- in maintenance of the proper volume of airway surface liquid (the periciliary liquid layer and the mucus layer that overlie the ciliated airway epithelium) in the respiratory tract has a consequent impact on the susceptibility of the tissue to infection, as illustrated, for example, by infections associated with cystic fibrosis (36–40). Importantly, Cl^- also plays fundamental roles in the biology of immune cells (41, 42). A prime manifestation of this is in the formation of hypochlorous acid (HOCl) by the myeloperoxidase (MPO) system, especially abundant in neutrophils (43). HOCl is formed via the reaction of hydrogen peroxide with Cl^- and H^+ , and is an extremely potent oxidant that plays an important antimicrobial role against various pathogens (43–47). HOCl has further been reported to play roles in the formation of neutrophil extracellular traps (NETs) (43, 48–50) and to act as an adjuvant, aiding induction of adaptive immunity (51, 52). Cl^- flux in neutrophils has been linked to granule release (53), and the activity of cathepsin C, which is key to activation of other proteases such as elastase and cathepsin G (54), is dependent on Cl^- binding (55–57).

The ability of Cl^- to act as a balancing ion is also critical for immune cell function. In particular, acidification of the macrophage phagosomal compartment during phagosome maturation is an extremely well-studied aspect of the maturation process, with pH a known critical cue for multiple pathogens (see for example references 58–63). However, the influx of H^+ must be simultaneously balanced by movement of other cations or anions to maintain electrical neutrality, such that H^+ movement is not prevented. It had previously been shown that in endosomes, Cl^- acts as a major counteranion during the acidification process (10), while efflux of K^+ had been described as occurring during lysosomal acidification (13). We demonstrated using both population-based and single phagosome-level assays that macrophage phagosomal acidification was inversely related to Cl^- concentration ($[\text{Cl}^-]$)—as pH decreases, $[\text{Cl}^-]$ increases, and disruption of acidification with bafilomycin A1 treatment results in loss of Cl^- accumulation (11). The $[\text{Cl}^-]$ within maturing phagosomes is ~ 70 to 95 mM on a population-level basis, with concentrations of >120 mM likely reached in some individual phagosomes (11). The lysosomal $[\text{Cl}^-]$ is ~ 108 mM (64), and CIC-7, a $2\text{Cl}^-/\text{H}^+$ exchanger, has been found to be critical in lysosomal biology (32, 65). Interestingly, mutation of *CLCN7*, the gene encoding CIC-7, was more recently reported to result in delayed macrophage

degradation of bacteria, with consequent inhibition of further phagocytosis (66). This feed-forward loop linking bacterial uptake and clearance was mediated by NF- κ B, with the loss of Cl^- transport and consequent decrease in endolysosomal calcium (Ca^{2+}) levels being key to the delayed bacterial degradation (66). As such, Cl^- has a fundamental role in multiple aspects of host biology that are critical to host-pathogen interactions.

Bacterial exploitation of Cl^- as an environmental cue. Just as bacteria respond to acidification of the phagosomal compartment (58, 59, 61–63), the simultaneous change in $[\text{Cl}^-]$ that occurs during macrophage phagosome maturation suggests that Cl^- could also be exploited as an environmental cue. *Listeria monocytogenes* escapes from the phagosomal compartment into the host cell cytosol where it replicates, and the bacterial toxin listeriolysin O (LLO) is vital in this process (63, 67–69). Oligomerization of LLO into a prepore complex is a first step in the lytic activity of LLO (70), and acidic pH had previously been reported to enhance LLO oligomerization and thus activity (71). Intriguingly, Radtke et al. discovered that LLO lytic activity was increased in the presence of high $[\text{Cl}^-]$, as was its oligomerization (72). While the identity of the exact channels/transporters that mediate changes in $[\text{Cl}^-]$ within the phagosome remains a matter of debate (73–75), inhibition or mutation of the CFTR channel decreased *Listeria* escape into the cytosol (72). Further, inhibition of CFTR prior to oral challenge significantly reduced systemic dissemination, with an ~ 100 -fold decrease in bacterial load in the livers and spleens of treated versus control mice (72). Importantly, inhibition of CFTR did not alter phagosomal pH (72), and thus the data together provide evidence for the use of Cl^- as an environmental cue by *Listeria* during host infection.

In the case of *Mycobacterium tuberculosis*, acidic pH has long been recognized as a critical environmental cue (58, 76, 77), with almost half of the bacterial genes whose expression are altered during initial macrophage infection no longer responsive if phagosomal acidification is blocked by administration of concanamycin A, a selective inhibitor of vacuolar H^+ -ATPases (58). We found that *M. tuberculosis* responded to increased environmental Cl^- levels in a concentration-dependent manner, with considerable overlap of genes present in the Cl^- regulon with those in the larger pH regulon (11). Even more strikingly, the transcriptional response of *M. tuberculosis* to the concurrent presence of high $[\text{Cl}^-]$ and acidic pH was highly synergistic, reinforcing the linked nature of these two signals (11). In this context and considering the findings with LLO from *Listeria* described above, it is plausible that the oligomerization and activity of LLO may similarly show synergistic enhancement in the presence of both acidic pH and high $[\text{Cl}^-]$. Indeed, a further finding of note supporting this idea is the increased stability of LLO at high $[\text{Cl}^-]$ (71).

Utilization of a fluorescent reporter *M. tuberculosis* strain where the promoter of a Cl^- /pH-responsive gene is used to drive green fluorescent protein expression (*rv2390c'::GFP*) allowed us to establish the changes in these environmental cues during infection (11). In particular, we observed increased reporter fluorescence, indicative of a higher $[\text{Cl}^-]$ and lower pH environment, during *M. tuberculosis* infection of activated versus resting macrophages (11). Similarly, reporter expression was higher during *M. tuberculosis* infection of wild-type than interferon- γ -deficient mice (11), which fail to properly activate macrophages and are unable to contain *M. tuberculosis* infection (78, 79). These data demonstrate transcriptional response of a bacterial pathogen to changes in environmental $[\text{Cl}^-]$ and pH, and highlight how the host immune response is linked to changes in the local ionic environment with regard to abundant ions.

Utilization of Cl^- as an environmental cue is not limited to intracellular bacteria sensing and response to $[\text{Cl}^-]$ changes during phagosome maturation. *Streptococcus pyogenes* (group A *Streptococcus* [GAS]) is extremely adept at colonizing various sites in the human host, resulting in disease manifestations that range from superficial skin infections to sepsis (80, 81). The expression of several GAS virulence factors has been reported to be affected by external $[\text{Cl}^-]$, with for example transcript levels of the multigene activator (*mga*) gene and genes responsible for streptolysin S production (*sag* genes) increased at high $[\text{Cl}^-]$ (82). Conversely, expression of other genes such as the

TABLE 1 Cl^- exploitation/modulation by pathogens

Organism(s)	Environmental $[\text{Cl}^-]$ change exploited or host Cl^- channel/transporter modulated ^a	Response/impact	Reference(s)
<i>Listeria monocytogenes</i>	Increased phagosomal $[\text{Cl}^-]$	Increases listeriolysin O oligomerization and activity; inhibition of CFTR reduces systemic <i>Listeria</i> dissemination	72
<i>Mycobacterium tuberculosis</i>	Increased phagosomal $[\text{Cl}^-]$	Transcriptional response that overlaps and is synergistic with response to acidic pH; reflective of host immune status	11
Group A <i>Streptococcus</i>	Increased environmental $[\text{Cl}^-]$	Increases expression of virulence factors (e.g., <i>mpa</i> and <i>sag</i> genes)	82
<i>Helicobacter pylori</i>	Hypochlorous acid (HOCl) production	Serves as a chemoattractant, potentially aiding bacterial sensing of tissue inflammation	86
<i>Pseudomonas aeruginosa</i> , <i>Acinetobacter nosocomialis</i>	CFTR	Bacterial Cif protein reduces host CFTR expression and thus Cl^- secretion across the epithelium	87, 88, 95
<i>Toxoplasma gondii</i>	CFTR, CaCC	Infection decreases Cl^- secretion via CFTR and the Ca^{2+} -activated Cl^- channel CaCC in airway epithelium	100
<i>Trichomonas vaginalis</i>	CFTR	Infection downregulates CFTR protein levels in vaginal epithelial cells	101
<i>Clostridioides difficile</i>	Epithelial disruption	Infection increases Cl^- secretion	102–104
<i>Entamoeba histolytica</i>	CFTR, CaCC	Infection stimulates CFTR and CaCC and increases Cl^- secretion	102, 103, 105
Enteropathogenic <i>Escherichia coli</i>	DRA	Infection inhibits Cl^-/OH^- exchanger DRA and redistributes it to intracellular compartments from the apical surface, increasing Cl^- secretion	102, 103, 106
<i>Vibrio cholerae</i>	CFTR, CaCC	Infection increases Cl^- secretion; cholera toxin and NAG heat-stable toxin activity stimulate CFTR, while accessory cholera toxin stimulates CaCC	102, 103, 107–109

^aOnly host Cl^- channels or transporters modulated are indicated. Note that several of the listed pathogens also alter expression or function of other host ion transporters (e.g., Na^+ -related transporters).

cysteine protease *speB* were decreased at high $[\text{Cl}^-]$. Intriguingly, significant overlap was observed in the transcriptional changes mediated by high $[\text{Cl}^-]$ (150 mM, with ~ 100 mM being a threshold point) and slightly basic pH (pH 7.5), indicating a link between these two signals (82). The pH of blood is normally maintained at ~ 7.4 (83), with $[\text{Cl}^-]$ being ~ 106 mM (84), and it is noteworthy that there is overlap among genes whose expression are altered during GAS growth in human blood (85) with those reported to change in the presence of pH 7.5 and high $[\text{Cl}^-]$ (82). This includes increased expression of *mga*, the *sag* genes, and several mannose phosphotransferase (*man*) genes (*manL*, *manM*, and *manN*), and decreased expression of genes such as *speB*, *dnaK*, and *dnaJ* (82, 85).

Finally, a recent study discovered that HOCl acts as a chemoattractant for *Helicobacter pylori*, with sensing of this signal accomplished via the bacterium's cytosolic chemoreceptor transducer-like protein D (TlpD) (86). TlpD was not sensitive to H_2O_2 or superoxide, precursors to HOCl formation, with HOCl instead directly affecting TlpD activity via oxidation of a conserved cysteine in the protein (86). *H. pylori* is resistant to high concentrations of HOCl, and the authors proposed that this sensing of HOCl as a chemoattractant could serve, together with other environmental cues, in aiding bacterial sensing of tissue inflammation and successful persistent colonization (86). Proteins with related domains in *Salmonella enterica* (the chemoreceptor McpA) and *Escherichia coli* (the diguanylate cyclase DgcZ) were also found to possess reactivity toward HOCl, opening the possibility that sensing of HOCl as an environmental cue may extend to other bacterial species beyond *H. pylori* (86).

It is important to note that the phenotypes found in the studies above were specifically attributable to changes in $[\text{Cl}^-]$ and not to changes in osmolarity or other counterions. Together, these studies illustrate how bacterial pathogens are able to utilize the various Cl^- concentrations in different locations within a host as an environmental cue, modulating their responses and aiding in adaptation and survival (Table 1). They also show the links and synergy between Cl^- and other environmental cues, such as

pH, that exist, which represent a further important facet for future examination (discussed in additional detail below).

Active modulation of host Cl^- flux by pathogens. In addition to exploitation of naturally occurring differences and changes in environmental $[\text{Cl}^-]$ within the host, several bacterial pathogens have been reported to actively modulate host Cl^- levels during infection. A key example of this is the CFTR inhibitory factor (Cif) protein from *Pseudomonas aeruginosa*. Cif was originally discovered as a secreted bacterial epoxide hydrolase that reduces host cell CFTR expression on the apical membrane and consequently Cl^- secretion across the epithelium (87, 88). In addition to direct secretion, Cif is also delivered to host cells via packaging in bacterial outer membrane vesicles (OMVs) (89, 90). Strikingly, delivery of Cif through OMVs was found to be extremely effective, with just 3 ng of Cif delivered via OMVs causing a decrease in CFTR expression on the host cell surface equivalent to that obtained with 50 μg of purified recombinant Cif protein applied directly to the host cells (90). In addition to inhibiting Cl^- secretion, Cif-mediated reduction of CFTR expression further impacts Na^+ levels, as CFTR also acts to repress activity of the epithelial Na^+ channel ENaC (91, 92). As such, a decrease in CFTR expression results in derepression of ENaC activity. Combined, these perturbations decrease airway surface liquid levels and alter the local environment to one that is more conducive to *P. aeruginosa* colonization (93, 94).

Cif homologs have since been identified in the genomes of *Acinetobacter nosocomialis* and *Acinetobacter baumannii*, with characterization of the *A. nosocomialis* Cif homolog demonstrating that the protein shares functional attributes of the *P. aeruginosa* Cif protein (95). Beyond CFTR inhibition, more recent work has uncovered a further role of Cif in disrupting inflammation resolution during *P. aeruginosa* infection (96). While it remains to be elucidated what the role(s) of Cif may be in the natural environmental niches of *P. aeruginosa*, the importance of Cif in *P. aeruginosa* virulence has led to several studies aimed at discovering and designing inhibitors of Cif function (97–99).

There have also been several reports of parasite-induced downregulation of Cl^- flux in epithelial cells. First, ATP-induced Cl^- secretion via CFTR and the Ca^{2+} -activated Cl^- channel (CaCC) in airway epithelial tissue was decreased in the presence of *Toxoplasma gondii* infection (100). While transcript levels of the purinergic receptor $\text{P2Y}_2\text{-R}$ were slightly increased upon *T. gondii* infection (100), the mechanism by which the parasite modulates Cl^- flux remains to be fully elucidated. Second, downregulation of CFTR protein levels, but not transcript expression, has been reported with *Trichomonas vaginalis* infection of vaginal epithelial cells (101). Parasitic cysteine protease activity was implicated as the cause of this downregulation, although the identity of the responsible cysteine protease is unknown (101).

Modulation of host Cl^- secretion has also been studied particularly in the context of enteric infections that cause diarrhea, with upregulation of Cl^- secretion often being observed (102, 103). This is seen in both bacterial and parasitic infections and includes infection with *Vibrio cholerae*, *Clostridioides difficile*, enteropathogenic *E. coli*, and *Entamoeba histolytica* (102–107). Several host ion channels and transporters are affected by these infections, with for example both increased CFTR activity and inhibition of the Cl^-/OH^- exchanger DRA (downregulated in adenoma) (Cl^- into the cell, OH^- out) reported with different infections, with changes often mediated by toxins or secretion system effectors produced by the pathogens (Table 1) (102, 103, 106–109). Cl^- is thus not simply a bystander ion during infection but one whose natural concentration changes are both exploited and modulated during infection by diverse pathogens (Table 1).

Based on sequence homology (for example, to mammalian CIC proteins), Cl^- channels and transporters are ubiquitous in the genomes of prokaryotes (19, 110). Despite their widespread presence, the biological function of these Cl^- systems remains largely unknown and unexplored. Just a few studies to date have addressed the biological role of CIC homologues in bacteria—a study in *E. coli* uncovered a role for its two CIC

homologues in extreme acid stress resistance (111), while studies in *V. cholerae* have similarly found a role for its CIC homologue in resistance to acidic pH (112, 113). With both *E. coli* and *V. cholerae*, the CIC homologues were found to be Cl^-/H^+ antiporters, similar to a subset of mammalian CIC proteins (19, 112–114). In the case of *V. cholerae*, deletion of its CIC homologue (*clcA*) also resulted in attenuation of colonization *in vivo* (112, 113). The importance of ClcA for colonization was dependent on location within the host, as while its activity aided bacterial survival in the stomach, a *clcA* mutant showed improved colonization in the intestine, and continued forced expression of *clcA* in the lower gastrointestinal tract was correspondingly detrimental for *V. cholerae* survival (112, 113). Further studies examining the role of Cl^- during infection and of Cl^- channels and transporters in bacteria and parasites will undoubtedly reveal new insights into pathogen biology.

POTASSIUM

Potassium is a critical signal in fundamental host cell processes and the immune response. Potassium (K^+) is the most abundant intracellular cation in mammalian cells, with concentrations in the 140 to 150 mM range intracellularly, versus 3 to 5 mM extracellularly (12, 14). Like Cl^- , K^+ plays a role in diverse fundamental host cell processes. In mammalian systems, this spans essential roles in muscle contraction and renal function, to information transmission in neurobiology and cell proliferation, with membrane potential regulation being a key function (12, 115–118). Numerous K^+ channels and transporters exist to permit the controlled movement of K^+ required for different processes, and there are diseases associated with disruption of K^+ channel/transporter function (119–122), as well as the development of pharmacological tools targeting these proteins (123–126).

In the context of infection, K^+ (like Cl^-) plays a significant role in the host immune response. Activation of the NLRP3 inflammasome occurs upon exposure to diverse stimuli, ranging from bacterial pore-forming toxins to particulate matter (127, 128). The cellular signal linking these varied stimuli was found to be K^+ efflux, with NLRP3 inflammasome activation triggered by low intracellular $[\text{K}^+]$ (129, 130). More recently, the identity of the K^+ efflux channel involved in this process of NLRP3 inflammasome activation in macrophages was reported to be TWIK2 (two-pore domain weak inwardly rectifying K^+ channel 2) (131). K^+ also acts in T-cell activation, via the activity of both voltage-gated K^+ channels, such as Kv1.3, and Ca^{2+} -activated K^+ channels, such as the IK channels (intermediate-conductance, Ca^{2+} -activated K^+ channels) (132–135). In addition, K^+ channels are involved in macrophage activation and proliferation (99, 136–138).

K^+ levels vary significantly intracellular versus extracellularly, and K^+ efflux had been shown to occur as a counter to H^+ movement during lysosomal acidification, with lysosomal $[\text{K}^+]$ measured at 50 to 60 mM (13). Efflux of K^+ was, however, not observed during macrophage phagosomal maturation (139). Instead, our experiments utilizing a K^+ -sensitive fluorescent compound covalently linked to silica beads, as well as a K^+ -responsive fluorescent reporter *M. tuberculosis* strain, showed the converse, with $[\text{K}^+]$ increasing as the phagosome matured (139). Changes in K^+ levels are thus location, host cell type, and process specific. These features, together with its important role in the host immune response, mark it as an ion with clear potential as an environmental signal and target for pathogens during infection.

K^+ as an environmental cue for bacteria and parasites. The stark difference in intracellular (high) versus extracellular (low) $[\text{K}^+]$ suggests the possibility of pathogen exploitation of K^+ as an environmental cue, and indeed, several studies in both bacteria and parasites have provided evidence for this. With the obligate intracellular parasite *T. gondii*, a decrease in $[\text{K}^+]$ upon host cell permeabilization has been implicated as a signal for parasite egress from host cells (140). This appears to work in concert with an increase in intraparasitic Ca^{2+} levels mediated by a parasite-encoded phospholipase C, which is required for egress to occur (140). Such a coupling of $[\text{K}^+]$ and $[\text{Ca}^{2+}]$ changes with alterations in parasite behavior has since been reported for *Plasmodium falciparum*, where a decrease in environmental $[\text{K}^+]$ also results in an increase in intraparasitic

Ca^{2+} levels, likewise mediated by a parasite-encoded phospholipase C (141, 142). In accord with these results, exposure of *Plasmodium berghei* and *Plasmodium yoelii* sporozoites to high $[\text{K}^+]$ has been reported to enhance parasite infectivity (143).

There are similarly key functional consequences for bacterial pathogens triggered by the bacterial response to changes in environmental $[\text{K}^+]$. The secretion of substrates via the bacterial type III secretion system (T3SS) is most commonly delineated into three sequential phases, with secretion of structural needle proteins (early substrates) first initiating assembly of the needle complex (144). Molecular ruler proteins determine the switch to secretion of translocators (middle substrates), which localize to the needle tip and are responsible for forming the pore in the host plasma membrane, when the needle has reached an appropriate length (144). Finally, gatekeeper proteins control the switch to secretion of effectors (late substrates) when host contact has been established (144).

Besides physical contact with a host cell, environmental cues shown to be able to mediate this switch from translocator to effector secretion are reflective of aspects specific to the host intracellular versus extracellular environment. The concept here is that insertion of the needle into the host cell opens a path to sensing of the intracellular environment by the extracellular bacteria, consequently changing the cues sensed and resulting in a switch to effector secretion (145–149). For example, low $[\text{Ca}^{2+}]$ has been shown to trigger the switch from secretion of translocators to effectors in *Yersinia* as well as several attaching and effacing bacterial pathogens (enteropathogenic and enterohemorrhagic *E. coli* and *Citrobacter rodentium*) (145–149). In the case of *Salmonella*, a change from acidic to neutral pH has been found to initiate the switch from translocator to effector secretion (62, 150). The host environmental cues exploited in the regulation of this process has more recently been expanded, with high intracellular $[\text{K}^+]$ discovered as a trigger for the switching from secretion of translocators to effectors for the T3SS 2 of *Vibrio parahaemolyticus* (151). While low $[\text{Ca}^{2+}]$ serves as a host signal for translocator to effector switching of the *V. parahaemolyticus* T3SS 1 (152), it did not significantly affect secretion from the bacterium's T3SS 2 (151). Instead, Tandhavanant et al. found that addition of 100 mM KCl, but not 100 mM NaCl, to the culture medium resulted in increased secretion of effectors and decreased secretion of translocators, without affecting protein production levels of the substrates (151). Indeed, inhibition of new protein synthesis by addition of chloramphenicol did not alter the increase in secretion of effectors and decrease in secretion of translocators observed in the presence of high environmental $[\text{K}^+]$ (151). Further, abolishment of the difference in intracellular versus extracellular $[\text{K}^+]$ by treatment of host cells with valinomycin and ouabain resulted in a significant decrease in secretion of T3SS 2 effectors, but not T3SS 1 effectors (151).

In addition to acting as a cue triggering the switch from secretion of T3SS translocators to effectors, environmental K^+ levels also influence bacterial gene expression, beyond simply affecting expression of K^+ uptake and efflux systems. For example, K^+ has been reported to be a key signal that can precipitate dysbiosis of the oral microbiome, with increased $[\text{K}^+]$ in the gingival crevicular fluid in severe periodontitis (153, 154). Elevated $[\text{K}^+]$ changed the relative abundance of bacterial species present in the oral microbiome and, separate from these composition changes, strikingly increased the expression of hemolysins (153). At the same time, alterations in environmental $[\text{K}^+]$ changed the host immune response, with higher external $[\text{K}^+]$ being correlated with higher tumor necrosis factor alpha (TNF- α) levels and lower expression of the antimicrobial human β -defensin-3 (153).

An increase in bacterial virulence factor expression upon exposure to high environmental $[\text{K}^+]$ has also been reported for *Salmonella*, with several effector proteins of the *Salmonella* pathogenicity island-1 (SPI-1) T3SS showing an increase in expression and secretion in the presence of added KCl to the medium (155). It should be noted, however, that addition of NaCl also resulted in increased expression and secretion of some SPI-1 T3SS effectors, although the strength of the effect compared to that observed

TABLE 2 Bacterial and parasitic exploitation of K⁺ as an environmental cue

Organism(s)	Environmental [K ⁺] change exploited	Response/impact	Reference(s)
<i>Toxoplasma gondii</i> , <i>Plasmodium falciparum</i>	Decreased intracellular [K ⁺]	In concert with intraparasitic [Ca ²⁺] increase, acts as a signal for microneme secretion and/or parasite egress from host cells	140–142
<i>Vibrio parahaemolyticus</i>	High intracellular [K ⁺]	Triggers switch of type III secretion system 2 from translocator to effector secretion	151
Oral microbiome	Increased gingival crevicular fluid [K ⁺]	Changes relative abundance of bacterial species present in the oral microbiome and increases hemolysin expression	153
<i>Salmonella</i>	Increased environmental [K ⁺]	Increases SPI-1 type III secretion system effector expression and secretion (also seen to various degrees with NaCl)	155
<i>Helicobacter pylori</i>	Increased environmental [K ⁺]	Increases expression of type IV secretion system effector CagA (also seen with increased [Na ⁺])	157
<i>Mycobacterium tuberculosis</i>	Decreased environmental [K ⁺]	Unique upregulated bacterial gene regulon; downregulated-gene list has overlap with genes upregulated during iron starvation and oxidative stress	139

with KCl varied depending on the effector (155). Separating the effects of K⁺, Na⁺, Cl[−], and osmolarity in this phenotype will thus require further studies. Along the same lines, while there has been significant work done examining the impact of salt on *H. pylori* biology due to the link between a high-salt diet and gastric cancer (156–160), few studies have attempted to determine the possible differential contributing effects of K⁺, Na⁺, Cl[−], and osmolarity on the phenotypes observed, with NaCl having been utilized exclusively. One study that did try to distinguish these components found that addition of KCl or potassium acetate also increased expression of the bacterium's type IV secretion system effector CagA, although to a "lesser extent than observed with NaCl" (157). Of note, addition of osmoequivalent concentrations of solutes such as sucrose and magnesium chloride did not affect CagA expression, ruling out osmolarity and Cl[−] as the factors in play and reinforcing the role of Na⁺ and K⁺ (157). The impact of a high-salt diet on host cell biology is additionally complex (161–165), and continuing studies are required for a complete and cohesive mechanistic understanding of how a high-salt diet affects *H. pylori* infection biology and results in an increased risk for gastric cancer.

In *M. tuberculosis*, we found that genes upregulated in the presence of low environmental [K⁺] represented a regulon largely distinct from that observed with other known major external cues (139). Intriguingly, there was overlap of the list of downregulated genes with those upregulated during iron starvation and oxidative stress (139). What this may imply for *M. tuberculosis* physiology and/or for possible connections between environmental K⁺ levels and iron levels awaits further analysis.

These studies demonstrate how the differences in [K⁺] inherent in host cell biology are exploited by bacteria and parasites, aiding in their adaptation and enabling successful colonization of the host (Table 2).

Bacterial K⁺ homeostasis plays important roles in host colonization. As with mammalian cells, K⁺ is also the most abundant intracellular cation in bacterial cells, and K⁺ uptake systems are ubiquitous in bacteria, with the Kdp and Trk systems being two of the best studied (166). Disruption of K⁺ uptake systems have been shown to result in attenuation of host colonization in multiple bacterial species, including *Salmonella*, *Staphylococcus aureus*, *H. pylori*, and *M. tuberculosis* (139, 167–169). While canonically K⁺ uptake has been known to play an important role in bacterial resistance to osmotic stress (166, 170), the impact of disruption of K⁺ homeostasis on further aspects of bacterial biology is just beginning to be revealed. In *Salmonella*, inactivation of the K⁺ uptake systems resulted in defects in secretion of effector proteins from the SPI-1 T3SS (167). This impact of disruption of K⁺ uptake systems on

TABLE 3 Role of bacterial K⁺ homeostasis in host colonization

Organism	Bacterial K ⁺ uptake system disrupted	Impact on host colonization	Reference
<i>Salmonella</i>	Kdp, Trk, Kup	Mutants exhibit defects in secretion of SPI-1 type III secretion system effector proteins and are attenuated for colonization of mice and chicks	167
<i>Staphylococcus aureus</i>	Ktr	<i>ktrA</i> mutant outcompeted by wild type in a murine bacteremia model	168
<i>Helicobacter pylori</i>	Kch	<i>kchA</i> mutant highly attenuated in a murine infection model	169
<i>Mycobacterium tuberculosis</i>	Trk	Mutant disrupted in response to other environmental cues, such as Cl ⁻ and pH, and attenuated for colonization of host macrophages and in a murine infection model	139
<i>Pectobacterium wasabiae</i>	Trk	<i>trkH</i> and <i>trkA</i> mutants attenuated in expression of virulence regulator RsmB, with consequent decrease in pectate lyase activity and tissue damage in potatoes	171
<i>Streptococcus mutans</i>	Trk2	Mutant exhibits defects in acid stress adaptation, membrane potential maintenance at acidic pH, and biofilm formation	172

bacterial virulence extends beyond mammalian infections. For example, in the plant pathogen *Pectobacterium wasabiae*, inactivation of the Trk K⁺ uptake system attenuates expression of RsmB, a noncoding RNA vital for the bacterium's virulence, with a consequent decrease in pectate lyase activity and tissue damage in potatoes (171).

Beyond roles in osmoadaptation, further aspects of bacterial adaptation to its local environment are also impacted by K⁺ uptake systems. Deletion of the Trk2 K⁺ uptake system in *Streptococcus mutans*, a bacterium found in the oral cavity, decreased its ability to adapt to acid stress, altered membrane potential changes in the presence of acidic pH, and also inhibited the bacterium's ability to form biofilms (172). Indeed, K⁺ was found to be essential for *S. mutans* biofilm formation, as wild-type bacteria were unable to form biofilms when grown in defined medium that lacked any K⁺ (172). The role of K⁺ and K⁺ uptake systems in bacterial biofilms was addressed in depth in a study in *Bacillus subtilis*, where K⁺ and the YugO K⁺ channel were shown to mediate a "wave of depolarization" across a biofilm that coordinated bacterial metabolic state within the biofilm community (173). This study intriguingly raised the concept of "long-range electrical signaling" in the context of bacterial communities and adds a further layer of complexity to how pathogens may utilize environmental ionic cues.

Unlike the canonical Kdp system described in bacteria such as *E. coli* and *Salmonella*, the Kdp K⁺ uptake system in *M. tuberculosis* does not appear to respond to osmolarity (139). Interestingly, we found that perturbation of *M. tuberculosis* K⁺ homeostasis via deletion of the Trk K⁺ uptake system disrupted the ability of the bacterium to respond to other environmental cues such as Cl⁻ and pH (139). This effect was independent of any changes in maintenance of intrabacterial pH or membrane potential, and points to the close links between bacterial ionic homeostasis and environmental ionic cue response (139).

Together, these studies demonstrate the role of bacterial K⁺ homeostasis in host colonization (Table 3).

CONNECTIONS BETWEEN IONIC SIGNALS

A theme that emerges from many of the studies described above is the extensive connections between pathogen exploitation of, and response to, abundant ionic signals. This encompasses synergism in the response to ions whose concentrations change in a coordinated manner, such as with *M. tuberculosis* response to pH and Cl⁻ in the macrophage phagosome (11), and pathogen response to one ionic signal resulting in changes in concentration in a different ion, such as with K⁺ and Ca²⁺ during *T. gondii* and *P. falciparum* egress from their host cells (140–142). It further includes disruption of homeostasis of one ion affecting bacterial response to other environmental cues, as we have shown with a Δ ceoBC Trk K⁺ uptake system mutant in *M. tuberculosis* (139). While the mechanism underlying the linking of

signal response has been elucidated in a few instances (e.g., phospholipase C-mediated increase in intraparasitic Ca^{2+} levels upon environmental $[\text{K}^+]$ decrease [140–142]), much remains unknown regarding how the different ionic cues are integrated and a coordinated pathogen response accomplished.

A direct way that ionic signals may be connected is via cotransporters (symporters or antiporters) or ion channels or transporters that are regulated by a second ion, such as Ca^{2+} . It is also possible for one ion channel/transporter to have effects on a second ion transport system, as is the case with CFTR inhibition of ENaC activity (91, 92). Many examples of cotransporters that encompass different combinations of ions exist in mammalian systems. This includes the Na^+/K^+ -ATPase pump, which is critical to the maintenance of ionic homeostasis and membrane potential in mammalian cells (174), $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporters (NKCCs), which are involved in multiple host physiological functions (175), K^+/Cl^- cotransporters (KCCs), with roles in NADPH oxidase activation in neutrophils (176), and $\text{ClC Cl}^-/\text{H}^+$ exchangers (19). In prokaryotes however, ion cotransporters are both less numerous and much less well understood (3, 177). While several classes of cotransporters present in mammalian systems are not present in prokaryotic systems, others, such as the $\text{ClC Cl}^-/\text{H}^+$ exchangers and Na^+/H^+ antiporters, are found widely (3, 19, 110, 177, 178). Microbial ion channels or transporters whose activity is regulated by a second ion, often Ca^{2+} , are also known to exist and include, for example, Ca^{2+} -regulated K^+ channels (179, 180). Here again however, little is understood of their role in microbial biology, with studies often focused solely on their structure, as a gateway for understanding mammalian systems (181). Studies aimed at understanding the biological functions of microbial ion channels and transporters thus remain an important piece of the puzzle in uncovering how ionic signals may be integrated in microbial systems.

What about coordination of an adaptive response to multiple ionic signals? Transcription factors play primary roles in directing the gene expression changes that occur in response to environmental signals (182–184), and are thus also principal candidates with regard to bacterial response to ionic signals. In *M. tuberculosis*, the two-component system PhoPR is required for the bacterium's ability to respond to acidic pH (58, 185, 186). As described earlier, we discovered that Cl^- also acts as an environmental cue for *M. tuberculosis*, with the bacterium responding synergistically to acidic pH and high $[\text{Cl}^-]$ (11). We found that PhoPR also plays an important role in the bacterium's Cl^- response, with a *phoPR* mutant exhibiting a decreased transcriptional response to Cl^- (11). Intriguingly however, the ΔphoPR mutant continued to show a synergistic response to the combined cues of acidic pH and high $[\text{Cl}^-]$, despite having no response to acidic pH alone (11). Excitingly, this indicates the presence of other regulators that contribute to the synergistic response of *M. tuberculosis* to acidic pH and high $[\text{Cl}^-]$. The response of a given two-component system to more than one environmental signal is also illustrated by systems such as DosRST in *M. tuberculosis* (hypoxia, nitric oxide, and carbon monoxide) (187–189) and KdpDE in multiple bacteria such as *E. coli* and *Salmonella* (K^+ and osmolarity) (190, 191). Besides transcription factors, the role of alternative sigma factors in bacterial response to abundant ions remains an open question. Additional layers of regulatory control and coordination are further likely to occur post-transcriptionally, via the action of small regulatory RNAs and serine/threonine protein kinases for example (192, 193). Examining the mechanisms by which microbial sensing of different abundant ion signals is transduced to adaptive responses will not only aid in understanding the fundamental role of these ions in microbial biology, but also open the door to the potential targeting of these responses in disrupting host colonization.

Overall, the findings of links between ionic signal sensing and response argue for the need to systematically examine microbial responses to these cues not just individually, but in combination, in order to understand when and where synergism

(or antagonism) may exist and to place these responses in the context of the infection/environmental biology of the specific pathogen.

CONCLUDING REMARKS

Despite, and perhaps because of, their prevalence, the role of abundant ions in microbial pathogenesis, with the exception of Ca^{2+} , has remained a largely unexplored area of inquiry. The evidence that abundant ions such as Cl^- and K^+ can and do play vital roles in microbial infection biology is however beginning to build, as highlighted in this review. The wide range of bacteria and parasites discussed above points to how widespread the phenomenon of microbial exploitation of abundant environmental ionic signals is likely to be. We are clearly just scratching the surface of how pathogens are able to exploit and usurp these signals, and many questions remain. How exactly are changes in the local concentrations of abundant ions such as Cl^- and K^+ sensed? How do these changes relate to other environmental signals, and how is the microbial response to these disparate signals coordinated? Might other pathogens known to respond to acidic pH also respond synergistically to changes in $[\text{Cl}^-]$, as is the case with *M. tuberculosis*? What is the natural context for the evolution and function of microbial factors such as Cif? While this review has focused on the context of infection, it should be noted that fluxes in the levels of Cl^- and K^+ also occur in environments such as soil (194–196).

What about the role of Na^+ , which is the most abundant extracellular cation in mammalian systems, with significantly lower intracellular concentrations? Studies on Na^+ in bacterial infections have predominantly focused either on Na^+ in the context of NaCl as a salt and osmolarity, with effects of Na^+ versus Cl^- versus osmolarity seldom distinguished, or on the role of Na^+ in the context of the flagellar motor (see for example references 197–199). There are, however, some hints that Na^+ may also serve as an environmental cue, as briefly noted above with *H. pylori*. *Campylobacter jejuni* infection has further been reported to decrease Na^+ transport via the epithelial Na^+ channel ENaC, although the molecular mechanism by which this occurs is unknown (200).

The investigation of abundant ions in the conceptual framework of their impact as environmental cues in microbial biology thus represents an exciting, open, and fecund field for further studies. One that is likely to yield significant insight into not just fundamental microbial physiology but also host-pathogen interactions, informing our understanding of how pathogens adapt and establish replicative niches and how we may perturb these processes to shift the balance of infection in favor of the host.

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