HHS Public Access

Author manuscript

Trends Microbiol. Author manuscript; available in PMC 2022 May 01.

Published in final edited form as:

Trends Microbiol. 2021 May ; 29(5): 441–457. doi:10.1016/j.tim.2020.08.001.

Molecular Evolution of Transition Metal Bioavailability at the Host–Pathogen Interface

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Abstract

The molecular evolution of the adaptive response at the host–pathogen interface has been frequently referred to as an ‘arms race’ between the host and bacterial pathogens. The innate immune system employs multiple strategies to starve microbes of metals. Pathogens, in turn, develop successful strategies to maintain access to bioavailable metal ions under conditions of extreme restriction of transition metals, or nutritional immunity. However, the processes by which evolution repurposes or re-engineers host and pathogen proteins to perform or refine new functions have been explored only recently. Here we review the molecular evolution of several human metalloproteins charged with restricting bacterial access to transition metals. These include the transition metal-chelating S100 proteins, natural resistance-associated macrophage protein-1 (NRAMP-1), transferrin, lactoferrin, and heme-binding proteins. We examine their coevolution with bacterial transition metal acquisition systems, involving siderophores and membrane-spanning metal importers, and the biological specificity of allosteric transcriptional regulatory proteins tasked with maintaining bacterial metallostasis. We also discuss the evolution of metallo-β-lactamases; this illustrates how rapid antibiotic-mediated evolution of a zinc metalloenzyme obligatorily occurs in the context of host-imposed nutritional immunity.

The Host–Pathogen Interface: A Struggle for Supremacy

An infection constitutes one of the major selective pressures that act on humans, and the host–pathogen interaction contributes to shape the genetic diversity of both organisms [1]. The general antagonistic paradigm suggests that, ultimately, the outcome of the encounter between host and bacteria is decided by the interplay between immune effectors that evolved

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to directly damage the microbial cell and countermeasures deployed by the pathogen to 'blunt' these host insults. However, this paradigm has been reconsidered in the context of the competition between host and pathogens for nutritional substrates, which enhances the impact of modest alterations in metabolism and nutrient sensing on the ultimate outcome of an infection [2]. Metal ions are unique examples of such shared nutrients since their essentiality pairs with their toxicity. Thus, bioavailability of every transition metal is tightly regulated in both bacteria and the eukaryotic host. Successful pathogens have evolved strategies to scavenge metal ions from host extracellular proteins that transport metal ions between tissues and organs [3], turning the human host into an accessible and rich reservoir of these nutrients. Consequently, host evolution is driven by a necessity to maintain the adapted bacterial population in check. This is illustrative of the Red Queen hypothesis, which claims that competing species continually evolve to maintain a level of fitness in a shared niche [3,4]. The inflammatory responses associated with innate immunity and further restriction of metal ions via metal-chelation is a process termed **nutritional immunity** (see Glossary) [5], which acts to prevent infections [3]. To preserve the physiological intracellular bioavailability of metal ions, pathogenic bacteria employ a systems-level response termed **metallostasis** that allows not only for metal scavenging, efflux, and intracellular sequestration, but also for ribosome remodeling and metabolic reprograming that occurs as a result of severe metal restriction.

Pathogen metallostasis and host innate immunity exert reciprocal selective pressures on one another that have shaped the proteins that function at this interface. On the host side, the core proteins of nutritional immunity can be grouped in three categories: (i) transporters that induce metal poisoning or starvation in intracellular pathogens (Figure 1A); (ii) multifunctional iron-transport proteins, and (iii) secreted multimetal-chelating immune proteins (Figure 1B). Bacterial systems-level responses against these host defensive proteins rely on transport systems specific to chemical speciation of metal nutrients in host tissues; tight regulation of metal acquisition systems across cell membranes is facilitated by the active transport of microorganism-derived metal-scavenging molecules, for example, siderophores and metallophores, and ultimately, a metabolism-level response to host-induced starvation (Figure 1). The highly complementary nature of this 'all-hands-on-deck' response enables pathogens to survive an ever-changing landscape of metal bioavailability within the infected host.

Recent advances in genome sequencing and gene synthesis have driven the development of powerful tools to assess protein evolution. These advances parallel a revolution in structural biology which has enabled the structure determination of large transmembrane protein complexes at atomic resolution, as well as developments in dynamics and thermodynamics approaches, to probe the molecular basis of specificity. Ancestral sequence reconstruction (ASR) might be the most important approach used to unveil how and when new functions appeared throughout evolution (Box 1). Furthermore, directed evolution assays and deep mutational scanning coupled to phylogenetic analyses allow for direct inference of selective pressures acting upon protein sequences (Box 1). In this review, we discuss the impact of evolutionary pressures responsible for the remarkable structural and functional diversity that continues to drive the 'arms race' over transition metal bioavailability in the infected host.

Evolution of New Functions That Impact Nutritional Immunity in Human Host Proteins

The primordial origins of effector proteins that anchor nutritional immunity in humans are diverse. Some are ancestral metal-binding proteins that have more recently acquired novel molecular traits used to activate the immune response, while others have ancestral roles as damage-associated molecular patterns (DAMPs), with specific metal-binding properties only recently acquired, enhancing their antimicrobial activities. Although most proteins involved in nutritional immunity are soluble, and secreted into the extracellular space, the survival and replication of intracellular bacterial pathogens inside infected macrophages leverages a set of transporters normally found in the plasma membrane that are requisitioned to intoxicate these bacteria with Zn^{II} and Cu or starve them for Fe, Mn^{II}, and possibly other metal ions [6] (Figure 1A). For example, the natural resistance–associated macrophage protein (NRAMP-1, SLC11) restricts Mn^{II} and other essential metals from intracellular pathogens, particularly those that replicate inside a membrane-enclosed compartment. Remarkably, NRAMP-1 shares the same molecular scaffold, metal coordination site, and metal specificity profiles with MntH, a cognate Mn^{II} importer and virulence determinant encoded by many human bacterial pathogens [7]. This suggests a common archaeal ancestor and highlights pathogen- and host-deployment of very similar tools to obtain these essential nutrients [8].

Interplay between Host Iron-Chelating Molecules and Bacterial Receptors

Upon infection, the innate immune system interfaces with systemic iron transport and increases the synthesis of several iron- and heme-binding proteins. One of these proteins is transferrin (Tf), a monomeric glycoprotein likely ubiquitous in metazoans, and namesake for the Tf protein superfamily. The exceptionally high affinity of Tf for Fe^{III}, and its role in nutritional immunity in vertebrates, is well documented and widespread [9]. Despite its high affinity, Tf must release the iron upon endocytosis of a transferrin receptor (TfR)–Tf_2 complex, the mechanism of which has only recently been explored [10,11] (Figure 2A). Although TfR is present only in vertebrates [12], an outer membrane-embedded transferrin receptor found in members of the families Neisseriaceae, Pasteurellaceae, and Moraxellaceae allows these bacteria to acquire Fe^{III} from Tf in a process termed ‘iron piracy’ [13]. The bacterial transferrin receptor is composed of two proteins, transferrin-binding proteins A and B (TbpA, TbpB), and each binds Tf at well defined, yet distinct interfaces [14,15]. This interaction with TbpA/B has likely driven Tf evolution, as suggested by the identification of positive selection sites in primate transferrins, located primarily in the Fe-binding C-lobe that binds TbpA, thus inhibiting that interaction, and blunting Fe piracy by the invading microbe [15] (Figure 2C). These positively selected sites are not expected to interfere with the binding of Tf to TfR, which primarily involves the N-lobe (Figure 2A); as a result, these Tf variants are expected to have little or no impact on systemic iron distribution in the host. This highlights an underlying evolutionary advantage of the bilobed architecture of Tf which accommodates a simultaneous evolutionary response to two orthogonal selective pressures, one derived from the host and the other away from the bacterial receptor (Figure 2C).

Lactoferrin (Lf) is another paradigmatic member in the Tf superfamily; it arose from a duplication of a primordial Tf gene in the ancestor of eutherian mammals (Figure 2B). Lf is a crucial component of the mucosal innate immune response within the granules of polymorphonuclear leukocytes, and its N-lobe has evolved new immunomodulatory properties that are independent of Fe binding (Figure 2B). The N-lobe is highly cationic and capable of disrupting the microbial membrane, while also harboring two regions that, when processed by host proteases, liberate peptides that exhibit potent antimicrobial activity (Figure 2B). Indeed, most of the residues undergoing positive selection in primates localize to the N-lobe, particularly in those regions that are precursors to antimicrobial peptides [16] (Figure 2C). Additionally, some sites in Lf under positive selection in the infected host negate a physical interaction with a bacterial inhibitory protein, for example, *Streptococcus pneumoniae* PspA or the iron piracy receptor *Neisseria* LbpB which specifically extracts Fe from the Lf N-lobe (Figure 2C). Overall, the evolution of the Tf superfamily in humans exemplifies how gene duplication and subfunctionalization dramatically alter the landscape of host–microbe conflict [4].

The observation that Fe-binding proteins constitute an important reservoir of genetic resistance to infectious disease is not exclusive to the Tf protein family, as recurrent positive selection on primate α- and β-globins restricts hemoglobin (Hb) binding and Fe acquisition by pathogenic *Staphylococcus aureus* in another form of iron piracy [17] (Figure 2D). Here, the human acute-phase protein haptoglobin (Hp) restricts the availability of Hb-bound Fe to the pathogen by physically blocking one of the two staphylococcal Hb-binding protein-mediated heme extraction pathways, by the iron-regulated surface determinant IsdH [18] (Figure 2D). Remarkably, Hp prevents bacterial heme extraction not by direct interaction with IsdH but by restricting the conformational ensemble of Hb, thus weakening the receptor interaction [19]. It is not yet known if the second staphylococcal Hb receptor IsdB appeared as a result of a duplication event in pathogenic *Staphylococc*us spp. to evade Hp-mediated inhibition of Fe uptake (Figure 2D), but it is known that IsdB is found exclusively in staphylococci capable of infecting deep tissues and causing invasive disease [17].

The Origins of the Antimicrobial Activity of Calprotectin

The innate immune system has also evolved modern proinflammatory proteins deployed to restrict a broader variety of transition metal ions beyond Fe^{III}. These proteins are members of the S100 superfamily of calcium-binding proteins found only in vertebrates and in their closest phy-logenetically related group [20] (Figure 3A,B). While S100 proteins have multiple intracellular functions [21], most are secreted into the extracellular milieu and bind pattern-recognition receptors (PRRs) which generally results in the initiation and upregulation of an inflammatory response, for example, the receptor for advanced glycation end products (RAGE) and Toll-like receptor 4 (TLR4) [21]. These receptors are well-known for their recognition of DAMPs or pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS). In contrast to what is considered an ancestral proinflammatory response characteristic of nearly all S100 subgroups, the calgranulin subgroup, composed of S100A7, S100A8, S100A9, and S100A12 in humans, has recently evolved a unique antibacterial activity defined by transition metal sequestration, while also retaining proinflammatory activity [20,22–24] (Figure 3B).

The four mammalian calgranulins likely arose as a result of gene duplication from an ancestral calgranulin; a fifth member of the calgranulin subfamily, sauropsid calgranulin (MPR126), is found only in birds and reptiles [20,23]. All five calgranulins harbor a conserved tetrahedral Zn^{II} coordination site, characterized by a low- to sub-nanomolar affinity, and exert some level of antimicrobial activity towards certain pathogens [25–28]. By contrast, the heterodimeric complex of S100A8 and S100A9 (named calprotectin, CP), and particularly in its Ca^{II}-induced $S100A8_2/S100A9_2$ tetrameric form, exerts potent antibacterial activity against a broader spectrum of pathogens [24,29,30]. CP is unique among calgranulins as it harbors an additional transition-metal coordination site 2 at the heterodimer interface, consisting of six histidines from S100A8 and S100A9 subunits (Figure 3A). Site 2 is largely metal-agnostic and conveys nanomolar or greater affinity for all late 3*d*-block divalent metal ions, Zn^{II}, Mn^{II}, Fe^{II}, Ni^{II}, Co^{II}, and Cu^{II} (Figure 3B). This allows CP to further restrict the growth of bacterial strains that harbor mechanisms to evade Zn^{II}-specific metal starvation induced by the other calgranulins [29,31,32]. Indeed, a recent ASR study (Box 1) reveals that only the ancestral CP conserves the hexa-histidine site and exhibits superior antibacterial activity, while the ancestral calgranulins show proinflammatory effects, albeit weaker than those of modern calgranulins [33].

Thus, the dual function of calgranulins requires two seemingly orthogonal characteristics: one as a short lived proinflammatory cytokine and the other as a protease-resistant transition metal-chelating protein. This apparent evolutionary conflict is elegantly resolved by the fact that S100A8 and S100A9 are each capable of forming both homodimers and the heterodimer CP. The antibacterial function is predominantly achieved by the Ca^{II}-bound heterotetrametric form of CP that is strongly resistant to degradation by proteases and binds TLR4 poorly [34,35]; by contrast, the relatively low stability of the S100A8 and S100A9 homo-oligomers enhances the temporal nature of an optimized proinflammatory response; indeed, this may have evolved through a single nucleotide polymorphism from a proteolysis-resistant, weakly proinflammatory ancestral calgranulin [33]. Emerging evidence suggests that CP and host-resident pathogens continue to coevolve with the discovery of a physical association growth-inhibition mechanism in *Borrelia burgdorferi* that is dependent on the ancestral Zn^{II} site, but independent of both metal restriction and the proinflammatory response [36]. On the bacterial side, a TonB-dependent transporter in *Neisseria* seems to have evolved to specifically capture human CP-bound Zn^{II} and transport the ion to the periplasm, overcoming Zn^{II} starvation [37,38]. In the case of *Neisseria gonorrhoeae* this is a specific adaptation to the human host as the bacterial receptor shows higher affinity for Zn^{II} bound to the hexa-histidine site of human CP than to mouse CP [37]. Furthermore, and as discussed later, metal chelation by CP induces evolutionary pressure on metallo-β-lactamases, which depend on Zn^{II} coordination to confer bacterial resistance to β-lactam antibiotics. It is not surprising that rapidly emerging, highly antibiotic-resistant bacterial strains possess mechanisms to overcome the effect of CP on the functionality of these enzymes (*vide infra*).

Lipocalins

The mammalian lipocalin-2 (Lcn-2; siderocalin, NGAL) functions in nutritional immunity and, like CP, not only restricts pathogen access to transition metal ions but also activates the

inflammatory response (Figures 1B and 4A). Lcn-2 restricts Fe^{III} by sequestering the archetypical circular *tris*-catecholate siderophore Enterobactin (Ent), produced by members of the family Enterobacteria-ceae for acquiring iron, characterized by the highest Fe^{III} affinity among all known siderophores [39]. Known siderophores are collectively characterized by an impressive diversity of chemical structures far beyond Ent that provide bacteria multiple ways to scavenge (and solubilize) this essential nutrient without directly interacting with human iron-transport proteins [40]. Additional host-driven evolutionary pressures may in fact have selected against Ent [41]. Consistently, hypervirulent bacterial strains encode multiple siderophores, and successful pathogens have leveraged an impressive diversity of biosynthesis pathways [42,43] and Fe^{III}-uptake mechanisms to enhance Fe acquisition and thus pathogenesis. A well known evolutionary adaptation against Fe^{III}–Ent sequestration by Lcn-2 is the modification of Ent through the addition of glucose groups to the C5 carbon to produce salmochelin (Sal) [44], which binds weakly to Lcn-2 (Figure 4A). Lcn-2 is also implicated in the regulation of the inflammatory response as it modulates the expression of cytokines in neutrophils and macrophages [45,46].

The lipocalin superfamily encompasses secreted proteins that bind a wide array of small hydro-phobic ligands with high affinity, despite low sequence identity, and perform a transport function in the vascular system of pluricellular organisms [47,48]. To the best of our knowledge, no phylogenetic studies have reconstructed an ancestral Lcn-2, so ancestral or derived biological functions cannot be inferred, as has been done with CP. However, there is emerging evidence that Lcn-2 has a host-protective function in kidney epithelial cells, beyond its known antimicrobial activity, by binding simple catechols secreted by gut commensal bacteria for endogenous iron transport in aseptic injuries [49]. This suggests that the antimicrobial activity of Lcn-2 may have evolved from an ancestral commensal interaction with a nonpathogenic enteric bacterium.

Rapid Evolution of Antimicrobial Resistance in the Context of Nutritional Immunity

Multidrug-resistant bacteria represent one of the major threats to modern human civilization, as antibiotic-resistance genes are spreading around the world at an alarming rate. Hydrolysis (inactivation) of β-lactam antibiotics by bacterial β-lactamases (BLs) is the most prevalent mechanism of resistance [50,51]. The extensive use and misuse of these life-saving drugs exert a strong evolutionary pressure on bacteria that results in the selection of BLs capable of providing enhanced resistance. The evolution of BLs uncovered by molecular epidemiology therefore reports on the different bacterial microenvironments that define evolutionary restraints (Box 1) [52]. BLs can be grouped into two large families: serine β-lactamases (SBLs) and zinc-dependent or metallo-β-lactamases (MBLs) [51,53]. While SBLs have been used as a model to study protein evolution, in particular TEM-1 [54–57], molecular evolution of MBLs is not fully understood and no MBL inhibitor has been identified or developed [58,59] for these rapidly evolving enzymes [60]. Our current understanding of SBLs cannot be translated to MBLs since these enzymes are evolutionarily unrelated, displaying different folds, active sites, and catalytic mechanisms [51]. MBLs are specifically affected by host nutritional immunity since Zn^{II} ions are essential for substrate

binding [61] and hydrolysis [62] (Figure 3A,C,D). MBLs belong to an ancient superfamily of Zn^{II} and Fe^{II} metalloenzymes with diverse activities, largely hydrolases and oxygenases [63]. There are three subclasses of MBLs (B1, B2, and B3) which differ in the Zn^{II} coordinating residues of the active site and Zn^{II} stoichiometries. Here we discuss the molecular evolution of B1 MBLs, the most extensively studied and clinically relevant among these enzymes. B1 MBLs bind two Zn^{II} ions in a conserved coordination motif (Figure 3C), where a Cys residue and second sphere ligands play key roles in tuning the Zn^{II} affinity [64,65].

Directed evolution experiments (Box 1) have provided significant insights into how mutations affect the catalytic efficiency and broad substrate specificity of MBLs [66,67]. Evolved variants of the *Bacillus cereus* MBL (BcII) that gain flexibility in the mobile loops flanking the active site display a broader substrate range than the native enzyme, without sacrificing the activity towards original substrates [68] (Figure 3C). This suggests that conformational dynamics is an evolutionary trait that can be optimized in MBL evolution (Box 2). These evolutionary lineages also allowed for the identification of an additional evolutionary trait, as evolved variants conferred enhanced resistance under conditions of metal starvation. Furthermore, strong **epistatic** interactions between coevolving residues dictate evolutionary outcomes under selective pressure, as mutations have **pleiotropic** effects, simultaneously impacting catalytic efficiency, protein stability, and Zn^{II}-binding affinity [69]. What is relevant here is that the Zn^{II}-binding affinity in the periplasm is an essential characteristic that defines the fitness landscape of MBLs.

New Delhi metallo-β-lactamases (NDMs) have recently arisen as potent, rapidly disseminating, broad-spectrum MBLs that show remarkable resistance to host-derived Zn^{II} starvation [70]. While most MBLs are readily degraded in the absence of Zn^{II}, NDM-1 accumulates in the periplasm in the apo form [71]. This is due to the fact that NDM-1 is lipidated at a Cys residue at the N terminus in a post-translational event after transport to the periplasm, which results in lipid anchoring to the outer membrane. Membrane anchoring renders NDM-1 resistant to degradation. This is a general phenomenon since engineering of the lipidation site into soluble MBLs also results in stabilization of these proteins in the absence of Zn^{II}. The soluble domain of NDM-1 has also been shaped by evolution to favor the interaction with the membrane. Thus, membrane anchoring is a novel mechanism by which MBLs can achieve resistance to metal chelation induced by CP [71,72] (Figure 3D). Remarkably, 29 allelic variants of NDM have now been reported in the clinic [73], and all accumulate mutations far from the active site; most confer enhanced resistance under conditions of zinc deprivation, relative to NDM-1 [74]. Although the specific effects and potential epistatic interactions of each mutation remain to be dissected, these mutations enhance resistance by increasing the Zn^{II} binding affinity, and/or augmenting the capacity of the apo forms to resist degradation in the periplasm, not by enhancing catalytic activity [74–76] (Figure 3D). The evolution of NDM β-lactamases represents a case study on how host-mediated nutritional immunity is driving the evolution of MBLs to enhance resistance to another pathogen challenge, antibiotics.

Evasion Mechanisms in Bacteria for Essential Nutrient Acquisition

A common feature of most successful pathogens is that they employ multiple, nonredundant and complementary mechanisms to acquire metal nutrients, notably Fe, to overcome host-mediated metal restriction. For example, while many streptococci possess only a single high-affinity Mn transporter [77], complementary mechanisms for Mn uptake in *S. aureus* have been shown to allow survival against the synergistic actions of nutritional immunity and other host defenses [78]. In *Vibrio* spp., different environmental signals allow for the expression of the more efficient Fe uptake mechanism in a certain niche [79]. Fe uptake mediated by siderophores constitutes a unique example of molecular evolution that shapes bacterial communities, due to the need to encode the biosynthetic pathways and/or the uptake systems required to utilize this form of Fe as a nutritional Fe source. Some organisms encode the capacity to biosynthesize more than one siderophore, which may be suitable for one host tissue over another, as recently observed in *S. aureus* in murine models of infection [80]. Other bacteria simply do not pay the biosynthetic costs to synthesize their own siderophores but instead express only the acquisition machinery, likely acquired via horizontal gene transfer, often for more than one siderophore class, in a process termed siderophore cheating. Other evasion mechanisms include the secretion or utilization of bacterially produced or host-endogenous *bis*- or monomeric Fe-chelating siderophores with lower Fe^{III} stabilities, for example, simple catecholamines or Ent hydrolysis products, that cannot necessarily outcompete Tf or Lf, but employ sacrificial redox chemistry [32] or enhanced cell permeability to acquire Fe from the surrounding tissues [81]. Mechanisms for the direct uptake of Fe^{II} are emerging in importance as a bacterial strategy that avoids competition with the host for Fe^{III} altogether; the host, in turn, is then able to deploy CP in an effort to sequester Fe^{II} from the pathogen (Figure 1B and Figure 4A).

Much of the chemical diversity of Fe^{III}-chelating siderophores derives from the evolution of different combinations of chain initiation, elongation/tailoring, and termination steps in non-ribosomal peptide synthetases (NRPS) [43]; in addition, four structural classes of NRPS-independent siderophore (NIS) synthetases that synthetize derivatives of citric acid are known [42]. The chemical diversity of these molecules is remarkable (with catecholate, hydroxamate, carboxylate, and mixed-type Fe^{III} coordinating donors); however, this diversity only allows for the uptake of Fe^{III}. Broad-spectrum metal-binding molecules, termed metallophores, employ a distinct biosynthetic pathway characterized by low levels of chemical diversity, at least as far as is currently known [82–84] (Figure 4B). The uptake of both siderophores and metallophores requires high-affinity membrane transporters to actively import these scarce nutrients at considerable energetic cost. In Gram-negative bacteria, nutrient import into the periplasm is mediated by porin-like transporters in the outer membrane (OM) that are coupled to the proton-motive force through interaction with the TonB complex, tethered to the inner membrane (IM). The ligand specificity of a TonB-dependent transporter (TBDT) is strictly dictated by the globular ‘plug’ domain and the extracellular loops of these 22-stranded transmembrane β-barrels [85] (Figure 4A). Once inside the periplasm, nutrients are imported into the cytoplasm by ATP-binding cassette (ABC) transporters, in which the periplasm-facing solute binding proteins (SBPs) bind the nutrient cargo; these tend to be more promiscuous than the TBDTs [86]. In Gram-positive

bacteria that lack an OM, the exquisite specificity of nutrient acquisition is dictated by the solute-binding lipoproteins themselves.

A suppressor screen in *Bradyrhizobium japonicum* mapped a handful of gain-of-function nutrient-adaptive SNPs and deletion mutations in a TBDT under conditions of iron deprivation and supplementation with a synthetic siderophore [87]. In other cases, the TBDT has some natural level of promiscuity, allowing transport of distinct endogenous and artificial siderophores [88]. TBDT plasticity derived from productive mutations or from functional group recognition also buttresses siderophore cheating, thus endowing the siderophore producer with a competitive advantage, while also serving a cooperative good [89]. TBDT plasticity may also constitute significant selective pressure to diversify siderophore biosynthesis pathways [90], as substantial fitness benefits of cooperation might not be evolutionarily stable [91]. In the context of an infection, **bacterial cheater** communities can be selected over siderophore producers [4], as has been demonstrated in *Pseudomonas* communities in lung tissues [92] (Figure 4B). The most extreme specialization is probably the synthesis, uptake, and production of membrane-anchored siderophores in *Mycobacterium tuberculosis* [91]. The evolutionary origin of siderophore secretion is unknown, but their soluble nature argues that, despite the self-directed benefits, the evolution of chemical diversity in siderophores may be determined in part by their indirect benefits to the community [91]. Future research in polymicrobial communities in the infected host will ultimately elucidate how the evolution of siderophore synthesis, secretion, and uptake has been shaped by both commensal and pathogenic bacteria [93].

Transcriptional Regulators Coordinate a Systems-Level Metallostasis Response

Metallostasis controls the expression of all nutrient acquisition processes described above in response to transition metal bioavailability, generally in the cytoplasm [94]. Both protein- and RNA riboswitch-based transcriptional regulation and post-transcriptional small regulatory RNA (sRNA)-mediated processes are operative [95,96] so as to avoid large fluctuations in metal levels, which could result in undermetalation or mismetalation of the proteome [31,97]. Protein-based transcriptional activators and repressors are bioinorganic allosteric switches and primary arbiters of bacterial metallostasis, as changes in gene expression result from changes in the metalation status of these **metallosensors**. Among the more than 20 structural classes of so-called ‘one-component’ sensors in bacteria, where direct ligand (metal) binding allosterically regulates DNA operator binding, no fewer than 12 have metallosensors [98]. Most metallosensors are helix-turn-helix and winged helix superfamily members proposed to be monophyletic [99], with the prominent exception of the CsoR (copper-sensitive operon repressor) protein superfamily, proposed to have evolved from a four-helix-bundle RNA-binding protein [100] (Figure 4C).

The capacity of bacterial pathogens to infect their hosts depends on the precise spatiotemporal regulation of gene expression, and it is this tight regulation that enables the evolution and diversification of the metallostasis response. Thus, the metal specificity and functional diversity of these metallosensors could serve as excellent model systems to

explore the molecular evolution of metallostasis. However, in most cases the evolutionary origin of transition metal specificity remains elusive, and almost certainly derives from contributions of the first (primary) and second metal coordination shells [100–102]. In addition, a high degree of sequence divergence over a relatively small domain structure (≈80–130 residues) hinders robust reconstruction methods that could be used to identify a primordial sensor for each metallosensor family. In many cases, even when sequence similarity networks suggest a common evolutionary origin [103], the structure of extant proteins with similar functional specificities, yet distinct metalloregulatory sites, suggests that many metal-binding sites in metallosensors may have convergently evolved [104] (Figure 4C).

As the bacterial genome size and developmental lifestyles increase in complexity, the number of individual members of a metallosensor family tends to increase [103,105,106]. Diversification of a metallosensor family may occur through gene duplication events that expand the evolutionary space of a protein without compromising an essential primordial function. In the case of MarR (multiple antibiotic resistance repressor), an ancestral family of transcriptional regulators that pre-dates the divergence of Bacteria and Archaea, gene duplication events are thought to have been the primordial means by which this family diversified [107]. On the other hand, horizontal gene transfer in polymicrobial communities has likely played an important role in the evolution of the metal-sensing motifs in metallosensors [103]. The metal specificity profile of a metallosensor is almost certainly evolutionarily linked to adaptation to a particular microenvironment, whether an obligate human pathogen, for example, *M. tuberculosis*, or an environmental organism, for example, *Methanocella arvoryzae*. For example, the actinomycetes *Streptomyces coelicolor*, a soil bacterium, and *M. tuberculosis* encode 13 and 10 arsenic repressor (ArsR) members, respectively, and 5 CsoR family members, far more than virtually any other organism. Moreover, the multiple metal-sensing sites of global uptake regulators for iron and zinc, Fur and Zur (ferric and zinc uptake repressors, respectively) [28], may define an evolutionarily acquired trait that enables a more nu-anced ‘graded’ response to distinct degrees of metal starvation, particularly important as Fur and Zur regulons expand [108,109].

Given the complications of studying the natural evolution of diverse metallosensor superfamilies, comparative studies of functionally distinct sensors from the same structural class have been undertaken to obtain ‘snapshots’ into the evolution of different biological outputs (functional diversity). In some metallosensor families new biological outputs are proposed to have arisen by missense substitutions that ‘remodel’ a single sensing site while maintaining an evolutionarily conserved allosteric connection(s) [101,105,110,111]. However, there are clear exceptions to this simple view. Cd^{II} sensing by *Pseudomonas putida* CadR (cadmium regulator), a MerR (mercury resistance regulator) family transcriptional activator [112], requires the unanticipated cooperation between a canonical thiolate-rich site I, remodeling of which generally impacts MerR family metal specificity, and a unique histidine-rich site II [113], thus repurposing a MerR into an exquisitely selective cadmium-sensing activator dependent on the evolution of a new allosteric connection. Similarly, in the Zn^{II} uptake repressor AdcR, a MarR family member from *S. pneumoniae*, the canonical ‘cleft’ that defines ligand recognition [105] and allosteric inhibition of DNA binding in other MarRs has been repurposed by the insertion of a flexible loop in the DNA-binding domain;

this evolutionary insertion entropically impairs DNA binding by apo-AdcR, and activates repression upon Zn^{II} coordination to primary and secondary metal sites, thus allowing for a more finely tuned 'graded' response to Zn starvation [114,115]. Finally, Cu^{I}-dependent inhibition and Zn^{II}-dependent activation of DNA binding in the copper sensor CopY from *S. pneumoniae* has evolved on the same molecular scaffold by repurposing the remarkable structural plasticity of BlaI, the β-lactamase repressor from *S. aureus* [116].

These examples seem to argue that bacterial pathogens have relied not only on the ability of allosteric systems to evolve new biology by permutation of a single ligand recognition site, but also the rapid evolution of new allosteric connectivities. A hallmark example of a metallosensor superfamily with diverse stressor recognition sites is the ArsR family of proteins (Figure 4C). ArsRs are characterized by the greatest metal-site diversity among all metallosensors in terms of the number of distinct sites and variety of metals, metalloids, and reactive small molecules that can be sensed by individual ArsRs. This functional diversity is anchored by at least five structurally distinct sensory sites that have evolved throughout a conserved molecular scaffold and that are proposed to have arisen from a single primordial Cd^{II} sensor (Figure 4C) [103]. Recently reported conformational dynamics experiments on a single ArsR protein reveal that allosteric connectivity is facilitated by an '**entropy reservoir**' that stabilizes the DNA-bound form of the repressor, and metal binding simply prevents access to this reservoir [98]. Although the degree to which these features characterize other ArsR family regulators is unknown, this model of evolution of new allosteric connections suggests that internal dynamics may create conserved latent allosteric connections, enabling new functionalities in the evolution of other metallosensor families [117] (Box 2). The existence of such latent allosteric connections within the physical scaffold of a functionally highly diverse metallosensor family suggests that it would be possible to rewire allostery of non-native inducer recognition sites in a directed evolution experiment [118,119], where the complications of natural evolution of an ancestral system are avoided.

Concluding Remarks

Microbial pathogens and vertebrate hosts engage in a perpetual struggle for supremacy. Nutritional immunity is among the first responses a pathogen encounters in a new host. Therefore, nutritional immunity has shaped, through natural selection, those bacterially derived molecules responsible for their virulence and adaptation to nutrient-deficient conditions. This adaptation has, in turn, resulted in selective pressures in host-derived proteins that function in nutritional immunity, which evolved new traits to counteract bacterial infections. Insights into this complex interplay, beautifully illustrative of the Red Queen hypothesis [4], have been gained only recently, with the application of directed evolution, ASR, and high-throughput sequencing methods. Here, we have highlighted this interplay in our discussion of the sequence variability of human iron-transport proteins, which originated with the evolutionary pressure from bacterial receptors that directly interact with them. Similarly, nutritional immunity exerts a selective pressure in bacterial metal-import systems resulting in diversification of siderophores by bacterial producers and in the emergence of new affinities of importers such as TBDTs from bacterial 'cheaters'. By contrast, the evolution of S100 proteins seems to have been driven by gene duplication

events that gave rise to a clade that evolved high-affinity transition metal-binding sites. In this area, ASR provided key insights into ancestral calgranulin function and helped to directly assess the emergence of new immune functions during evolution. We believe that this approach could yield breakthroughs in our understanding of not only the evolution of our immune response but also in our understanding of bacterial resistance and its emergence, particularly in the evolution of bacterial metalloregulators. Because of this, we hope to see ASR applied more consistently across the field. We believe that the evolution of regulation and antibiotic resistance in the context of nutritional immunity defines an exciting new field of research ripe for discovery, as protein dynamics and allostery have likely facilitated the rapid rise of new functionalities in bacterially encoded regulatory proteins [98,114] (see Outstanding Questions). As bacterial resistance becomes a major threat to humankind, understanding the molecular evolution of the systems reviewed here will play a critical role in preventing deadly pandemics in the postantibiotic era.

Acknowledgments

This work was supported by grants from the US National Institutes of Health (R35 GM118157 to D.P.G., R01 AI100560 to A.J.V.), and the Pew Foundation, USA, Bunge & Born, Argentina, and Williams Foundations, Argentina (to D.A.C.). D.A.C and A.J.V. are Staff Members from CONICET. G.T.A. is supported by a graduate fellowship provided by CONICET, Argentina.

Glossary

Bacterial cheaters	non-siderophore-producing bacteria that benefit from the siderophores synthesized by other bacteria (called xenosiderophores)
Entropy reservoirs	a set of residues in a protein that gain degrees of freedom (increased conformational entropy) upon a binding event, for example, in transition from an apo to a ligand-bound state
Epistasis	a generic term used to define a context-dependent effect of a particular mutation. Hence, the fitness effect of such mutation depends on the genetic background in which is introduced, that is, the presence or absence of another mutation
Metallosensors	allosteric transcriptional regulators that bind a specific transition metal(s) in a way that modulates DNA binding affinity and/or the expression of regulated metallostasis genes
Metallostasis	a systemic process by which cells control the intracellular bioavailability of functionally required transition metal ion cofactors, avoiding both metal ion starvation and toxicity. It is an abbreviated expression for bacterial ‘transition metal homeostasis’

Nutritional immunity	host innate immune response that involves the withholding of metal nutrients to prevent bacterial growth. One of the most relevant nutritional immunity proteins is calprotectin, which sequesters virtually every transition metal available at an infection site
Pleiotropy	a phenomenon in which a single mutation is responsible for affecting more than one molecular trait in a protein or enzyme, for example, protein stability and transition metal affinity

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Highlights

The interaction between human iron-transport proteins and bacterial receptors acts as a driving force for the diversification of both families of proteins.

Calprotectin, a pivotal nutritional immunity protein, evolved from a family of proinflammatory proteins that developed high-affinity binding sites for transition metals.

Plasticity of siderophore importers allows for nutritional iron acquisition by bacterial cheaters, which, in turn, selects for diversification of siderophore biosynthetic pathways in producers.

Comparative studies of inorganic sensors from the same structural class provide insights into the evolution of functional diversity in regulators of metallostasis.

Zn^{II}-binding affinity and apo-protein stability of periplasmic metallo-β-lactamases are key evolutionary traits of these enzymes in the context of nutritional immunity.

Box 1.

Tools for Assessing Protein Evolution

Identifying function-altering mutations in proteins is important because these can confer pathogen adaptability and evasion of the immune response of its host. A protein of interest can be compared with homologous proteins with different functions through horizontal analyses. Here, polymorphisms in residues important to catalysis are swapped between proteins, and functional shifts are experimentally assessed [120]. However, this approach usually fails because neutral substitutions occlude relevant ones, and epistatic interactions are not considered [120]. On the other hand, vertical analyses, like ASR, allow one to focus on substitutions that occurred when function changed. ASR is the calculation of ancient protein sequences using extant ones [121]. It is based on the phylogenetic analysis of a protein family obtained through multiple sequence alignments and a probabilistic model of evolution [120,121]. From this, ancestral protein sequences at every internal node of the phylogenetic tree can be inferred and artificially synthesized to evaluate changes in biological function during evolution. This has been beautifully illustrated by the Harms group for calgranulins and calprotectin [33]. By introducing mutations in the historical background where they occurred, ARS overcomes epistasis [120].

Multiple sequence alignments of extant proteins from evolutionarily divergent organisms allows us to quantitatively establish the presence of selective pressures acting upon a protein sequence. Positive selection is assessed by the estimation of the ratio between nonsynonymous (N) and synonymous (S) substitutions (dN:dS) across lineages [122]. Cross-analysis of dN:dS ratios between host-encoded and pathogen-encoded proteins is useful for identifying coevolving sites and causalities for natural selection [16].

Finally, artificial evolution methods uncover evolutionary pathways that lead to pathogen adaptation in hostile environments. All directed evolution approaches are based on expanding the sequence space of a given protein (through targeted or random mutagenesis), selecting for a desired phenotype, and allowing the genetic variants to replicate [123]. After multiple rounds of functional selection, it is possible to isolate a protein with new or optimized functionality [123]. In deep mutational scanning, the sequence space of a given protein is expanded in a high-throughput manner, as the functional consequences of every possible amino acid substitution are assessed for each position of a given protein. Necessarily, this requires a robust functional screening methodology [124]. In continuous evolution approaches, gene diversification, selection, and replication are carried out with minimal researcher intervention in continuous dilution systems [125]. Alternatively, the rapid evolution of a family of proteins, as the organism disseminates in new environments, can also be studied through molecular epidemiology, as in the case of β-lactamases [60].

Box 2.

Evolution in Motion: The Role of Dynamics in Protein Function

The sequences, structures, and functions of modern proteins are the result of an evolutionary process exerted upon sequence–structure–function space. Mutations constantly erode protein sequences, generating variants that may affect the biological activity of a protein, subjecting it to natural selection. An understanding of how changes in protein sequence are ultimately capable of generating new functions remains an elusive goal. The ‘one structure, one function’ paradigm suggests that function-shifting mutations should induce a measurable change in protein structure. However, this vision is rendered obsolete by the striking divergence in functions observed in many families of structurally homologous proteins. The native state of a protein is more accurately represented by an ensemble of conformations accessible at thermal equilibrium. This distribution of accessible states is encoded in protein dynamics. Changes in protein sequences impact on this equilibrium by restricting conformations necessary for their functions. Thus, altering the intrinsic dynamics of a protein is a mechanism by which new functions evolve.

Recent convergent developments in NMR spectroscopy, X-ray crystallography, and computational methods open up the possibility of merging structure and dynamics to obtain true atomic-resolution integrative descriptions of biomolecules [126]. Protein dynamics play a fundamental role in the most basic requirement for protein function: the capacity to bind to a particular ligand to the exclusion of all others. This tuning is generally difficult to accomplish from evolutionary perturbations of the few amino acids that directly interact with the ligand. Thus, specificity of a protein is controlled through conformational changes in groups of residues far beyond the ligand-binding site, allowing proteins to evolve allosteric connectivities and adapt to new selective pressures [127]. This is evidenced by multiple studies performed on β-lactamases and transcriptional regulators, where mutations occurring at sites far from the active site are responsible for altering protein function with minimal impact on the conformational ensemble [69,98,114]. Furthermore, this has been observed in directed evolution assays, where distant substitutions rescue native-like protein dynamics and restore enzymatic activity on functionally impaired proteins [67]. A recurring mechanism that seems to be employed by evolution of protein function is one that involves the ‘tuning’ of protein dynamics, or entropy redistribution. By enhancing the flexibility of certain sites while stiffening others, proteins retain the same overall fold but these substitutions impact their function, allowing either the acquisition of new functions or allosteric modulation of their activities [98,114,128].

Outstanding Questions

The emergence of a transferrin paralog allowed for accumulation of mutations that led to the antimicrobial activity of lactoferrin. Did gene duplication events also drive the emergence of new variants of bacterial hemoglobin receptors such as IsdB, which confers enhanced pathogenicity in *Staphylococcus* spp.?

Heterodimeric calprotectin acts as a potent antimicrobial transition metal chelator that forms tetramers of high stability, while S100A8 and S100A9 homodimers function as proinflammatory cytokines that are rapidly degraded by proteases. This prevents substantial damage to the host during inflammation. How is the production of these distinct complexes modulated at infection sites to maximize a systemic antimicrobial response?

The functional importance of an ‘entropy reservoir’ has been documented for the staphylococcal zinc efflux repressor, CzrA. Is the entropy reservoir a conserved evolutionary feature of the arsenic repressor (ArsR) protein family? If so, to what extent is this feature responsible for the emergence of new biological outcomes in this ubiquitous protein family?

What is the mechanism by which mutations far removed from the catalytic dinuclear Zn^{II} site in metallo-β-lactamases enhance the Zn^{II} binding affinity?

Why does membrane association provide enhanced periplasmic stability to apo-metallo-β-lactamases?

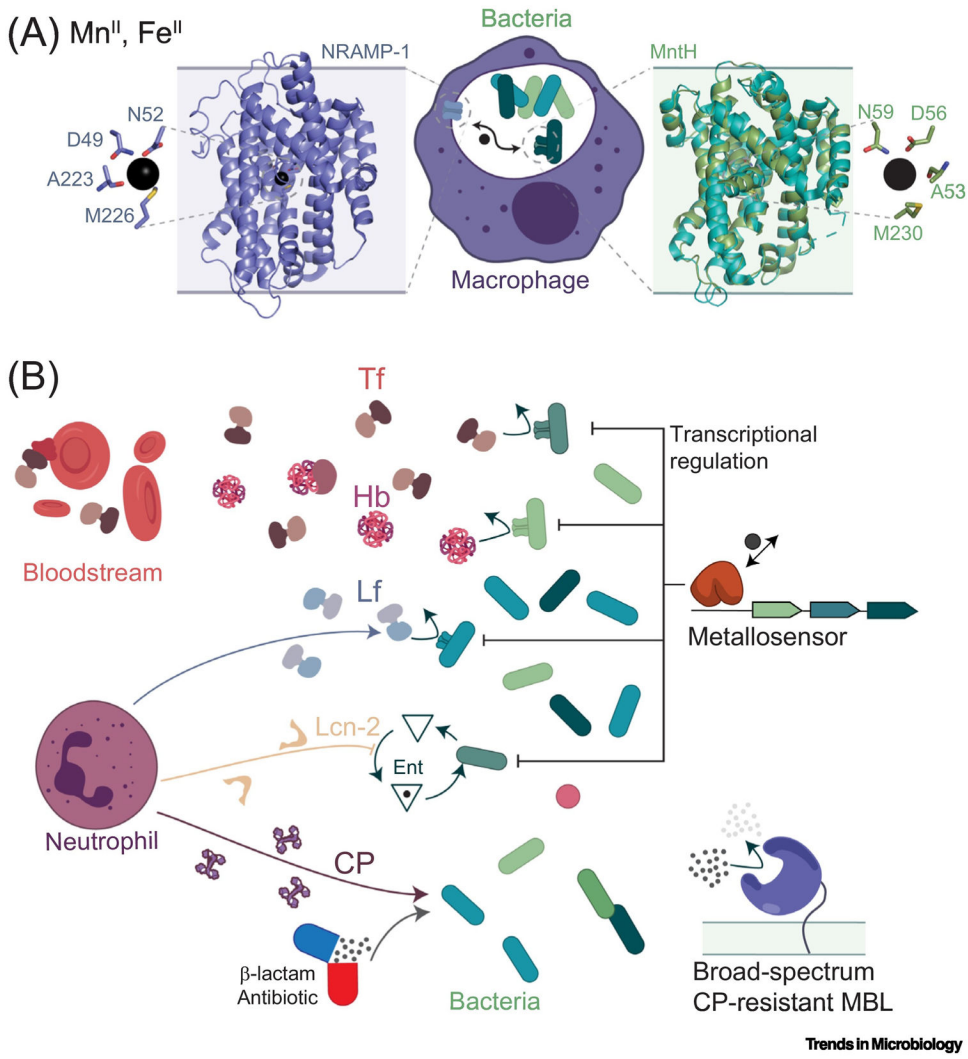

Figure 1. Schematic Illustration of Human Proteins Involved in Depletion of Essential Metal Ions during Nutritional Immunity (Both Panels, Left), and Bacterial Effectors of Metal Ion Acquisition and Metallostasis (Both Panels, Right).

Proteins are shown in cartoon representation, metal ions as black spheres, and cell membranes as shaded rectangles. (A) Intracellular pathogens' nutritional immunity proteins exemplified by the model human natural resistance-associated macrophage protein-1 (NRAMP-1) transporter DMT1 (*Staphylococcus capitis* DMT, PDB: 4WGW) and bacterial response exemplified by Deinococcus radiodurans MntH (PDB: 5KTE). Insets, Mn^{II} coordination chemistry. (B) Interplay between extracellular nutritional immunity proteins and bacterial response. Bacterial receptors for human serum transferrin (hTf), haptoglobin (Hb) and neutrophil-produced lactoferrin (Lf) drove the selection of variants with decreased affinity for the bacterial receptors or new immunomodulatory properties (curved arrows). Bacterial expression of metallophore production (exemplified by siderophore enterobactin or Ent) and import systems transcriptionally modulated by metallosensor proteins are counteracted by human neutrophil-produced lipocalin-2 (Lcn-2, also called siderocalin) and the multimetal-chelator calprotectin (CP). The latter, in addition to the use of β-lactam

antibiotics, drove the selection for broad-spectrum metallo-β-lactamases (MBLs) from highly resistant pathogenic bacteria.

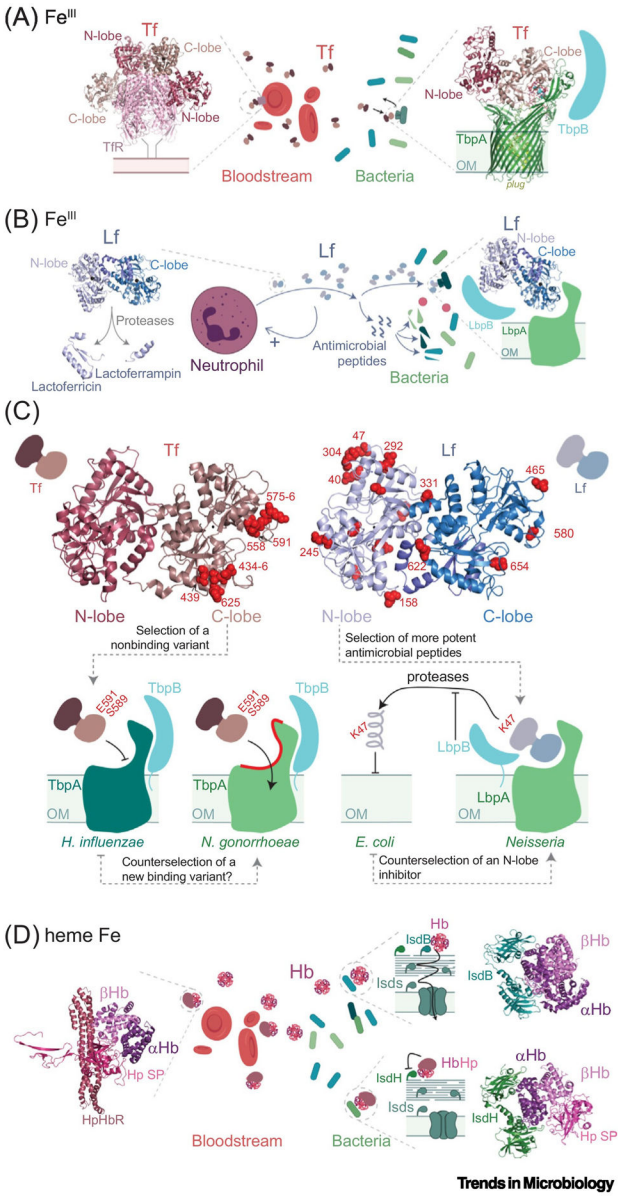

Figure 2. Selection of Human Iron Transport Proteins and Bacterial Uptake Systems.

Proteins are shown in cartoon representation and cell membranes as shaded rectangles. (A) Human transferrin (Tf, PBD: 1SUV) interacts through distinct interfaces (N-lobe or C-lobe) with its cognate receptor in erythrocytes (red cells) and the *Neisseria* receptor TsbA (shown in complex with Tf, PBD: 3V89) and TsbB. (B) neutrophil-produced lactoferrin (Lf, PDB: 1B0l) with the antimicrobial peptides produced by proteolysis, and bacterial response exemplified by LbpAB [16]. (C) Above: positively selected sites across the primate clade represented as red spheres on human serum transferrin (hTf, PDB: 3V83) [15] and human lactoferrin (hLf, PDB:1B01) [16]. Below: schematic representation of the interaction of these proteins with bacterial Tf receptors in iron piracy, highlighting the location of key selected sites on both proteins that either prevent binding to the bacterial receptor (Tf) or convey antimicrobial activity against certain pathogens (Lf). The dashed arrows between

bacterial species indicate an increased relative fitness. (D) Hemoglobin (Hb, in complex with haptoglobin, PDB: 4WJG) and bacterial response exemplified by *Staphylococcus aureus* receptors (IsdH, PDB: 6TB2 and IsdB, PDB: 5VMM), where the HbHp complex inhibits the bacterial receptor. Abbreviations: *E. coli*, *Escherichia coli*; *H. influenzae*, *Haemophilus influenzae*; *N. gonorrhoeae*, *Neisseria gonorrhoeae*; OM, outer membrane of Gram-negative bacteria.

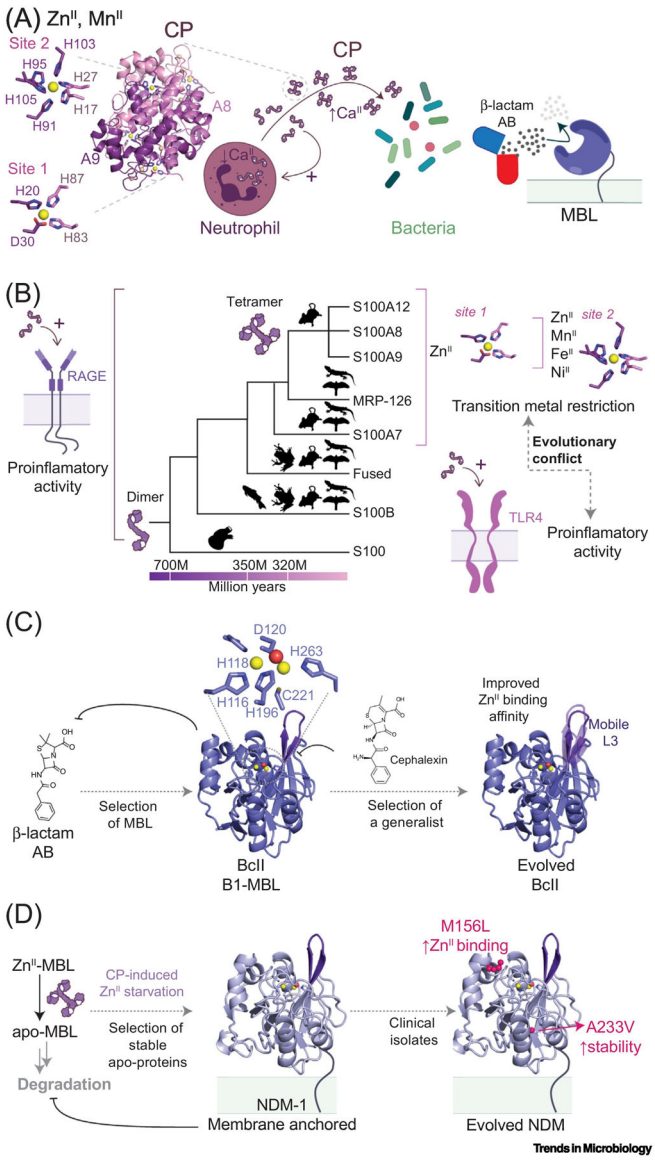

Figure 3. (A) Model of Action for Neutrophil-Produced Multimetal-Chelating Calprotectin (CP, PDB: 4GGF).

Dimeric CP is secreted to the extracellular milieu upon activation of neutrophils where high Ca^{II} levels induce heterotetramerization [30], which enhances transition metal affinity and resistance to extracellular proteases. Details of the transition metal coordination site 1 (Zn^{II}-specific) and site 2 are shown to the right. Left: β-lactam antibiotics and bacterial response exemplified with metallo-β-lactamases (MBLs), which are sensitive to Zn^{II} chelation by CP. (B) S100 protein evolution across vertebrates. They arose in the last common ancestor of vertebrates (represented by the fish, frog, mouse, bird, and lizard silhouettes) and urochordates around 700 million years ago, while calgranulins appeared in the ancestor of amniotes (320 million years ago). In mammals, the calgranulin clade expanded via gene duplication events (top) [20,23]. Illustration of conserved interaction of most S100 proteins with the receptor for advanced glycation end products (RAGE) (left) and calgranulin-specific interaction with TLR4, exemplified by heterodimeric CP and homodimeric S100A8

and S100A9 (bottom, right) [21,23]. Illustration of nutritional immunity activity restricted to calgranulins, with heterotetrameric CP eliciting broad-spectrum metal restriction attributed to a metal-agnostic binding site 2 that is unique in the S100 superfamily (top, right). (C) and (D): Evolution of MBLs. Proteins are shown in cartoon representation and the Zn^{II} ions as yellow spheres and coordinated water molecule as a red sphere. (C) The extensive use of β-lactam antibiotics acted on bacteria as a selective pressure that resulted in the expansion of MBLs in bacterial populations, such as BcII (PDB: 4NQ4). BcII cannot hydrolyze carbapenem and is sensitive to Zn^{II} depletion, as apo-MBLs are readily degraded by proteases in the periplasm. Further selection exerted by carbapenem antibiotics and nutritional immunity results in variants with increased antibiotic binding site flexibility, which enhances substrate promiscuity, and increased Zn^{II} binding affinity [61,68]. (D) In NDM-1 (PDB: 5ZGZ), membrane anchoring prevents proteolysis in the periplasm of the apo-form and renders the protein highly resistant to nutritional immunity-imposed Zn^{II} restriction [71,72]. Further mutations from clinical isolates (two are shown) that occur from the active site of the protein enhance resistance toward host-imposed Zn^{II} deprivation [74].

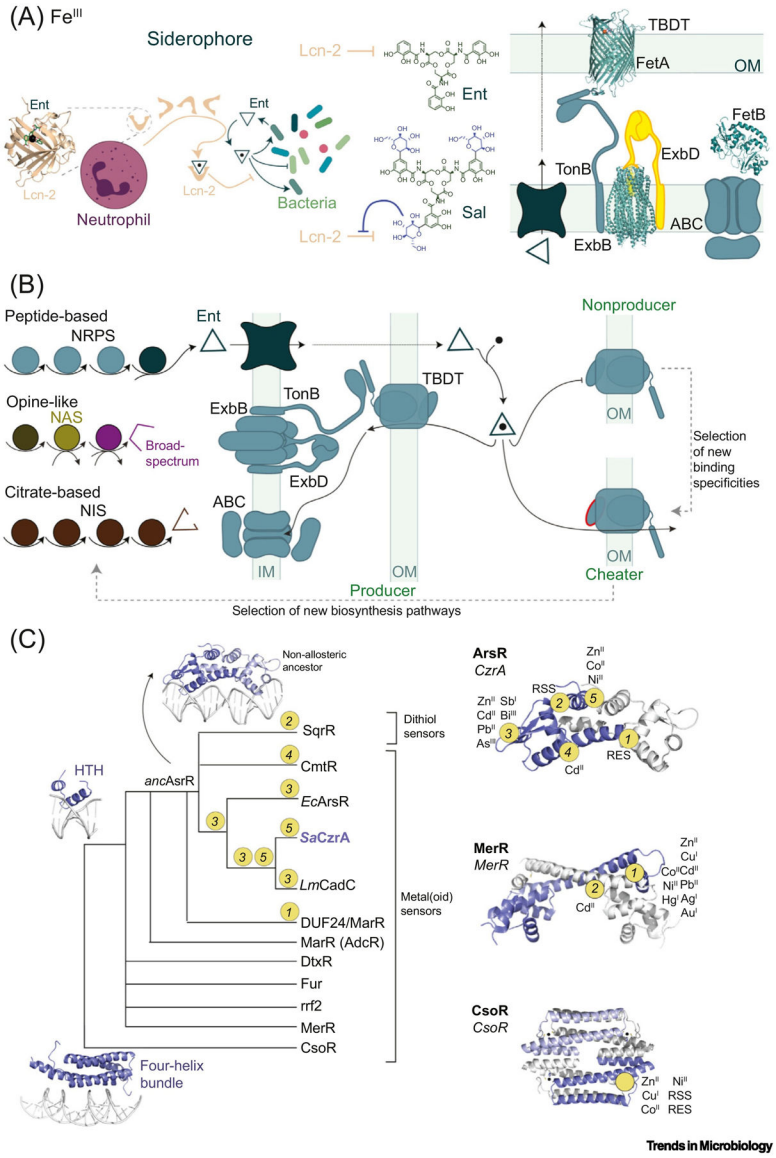

Figure 4. (A) Left: Neutrophil-Produced Siderocalin (Lcn-2, in Complex with Enterobactin, Ent; PDB: 3K3L) and Bacterial Response Exemplified with the Production of Ent and Salmochelin (Sal), Which Is Not Recognized by Lcn-2.

Right: Gram-negative bacterial import system exemplified by the TonB-dependent transporter (TBDT) ZnuD (PDB: 4RVW), ExbB/ExbD complex (PDB: 5SV1), and an ATP-binding cassette (ABC) transporter (showing the structure of solute-binding protein FepB (PDB: 2M6L). (B) Siderophore or metallophore production (left) and cellular uptake (right). In Gram-negative bacteria, siderophores are imported by TBDTs into the periplasm and then into the cytoplasm by ABC transporters. In non-siderophore-producing bacteria (cheaters), new binding affinities and specificities in TBDTs are selected for, allowing for adaptation to new niches [87]. This, in turn, exerts a selective pressure on siderophore-producing bacteria, resulting in new biosynthesis pathways that expand siderophore diversity. (C) Schematic representation of the evolution of transcriptional regulator families, as suggested by some studies [99,100,106,129]. Apart from the CsoR superfamily (which may have arisen from a

four-helix bundle ancestor), most metallosensors share the helix-turn-helix (HTH) topology in a common ancestor. In the arsenic repressor (ArsR) superfamily, the five sensory sites evolved from a nonallosteric ancestor with distinct metal or reactive species affinities and reactivities, respectively. The approximate positions of inducer recognition sites and sensing specificities are shown in representatives of the ArsR (2M30), MerR (5CRL), and CsoR (4M1P) superfamilies [103,130]. Abbreviations: NIS, NRPS-independent siderophore; NRPS, non-ribosomal peptide synthesis; OM, outer membrane; RSS, reactive sulfur species; RES, reactive electrophile species.