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Emerging Role of Ferrous Iron in Bacterial Growth and Host-Pathogen Interaction: New Tools for Chemical (Micro)Biology and Antibacterial Therapy

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Abstract

Chemical tools capable of detecting ferrous iron with oxidation-state specificity have only recently become available. Coincident with this development in chemical biology has been increased study and appreciation for the importance of ferrous iron during infection and more generally in host-pathogen interaction. Some of the recent findings are surprising and challenge long- standing assumptions about bacterial iron homeostasis and the innate immune response to infection. Here we review these recent developments and their implications for antibacterial therapy.

Keywords

Gram-negative bacteria; host-pathogen interaction; iron metabolism; iron speciation; labile iron pool; activity-based probes; reactivity-based probes; anti-bacterials; anti-infectives

Background

Antibiotics were the formative products of the early pharmaceutical industry, and their importance to human health has only increased in recent years [1]. The antibiotic ‘golden age’ of the mid 20th century saw unprecedented productivity in terms of numbers of new agents brought to market, but the focus on natural product scaffolds for which resistance mechanisms were already extant in bacterial populations, combined with rampant overuse of some agents, has led to the current situation where ~70% of bacterial pathogens exhibit resistance to the most widely used antibiotics [2,3]. It is estimated that by 2050, if no corrective action is taken, these infections will cause over 10 million deaths per year, with economic costs of 100 trillion USD in lost global production [4].

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Declaration of competing interest

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The dearth of effective treatment options is especially concerning for multidrug-resistant (MDR) Gram-negative bacteria such as carbapenem-resistant *Acinetobacter*, *Pseudomonas* and *Enterobacterales* [5]. These MDR pathogens possess efflux transporters and outer membrane permeability barriers, which act synergistically to increase their resistance to diverse antibacterial classes [6]. Horizontal gene transfer furthermore enables these pathogens to rapidly acquire new resistance mechanisms that further limit antibiotic efficacy [7]. This situation has led to calls for novel targets and new approaches that could lead to a more robust and less vulnerable antibiotic pipeline for the new century [8]. We argue here that increasing understanding and appreciation for the role of low-molecular mass ferrous iron species ('labile iron') in bacterial growth, pathogenicity, and host interaction could provide inroads into novel antibiotic strategies. The recent development of chemical probes of labile iron should aid such efforts but have to date been employed primarily in studies of eukaryotic iron homeostasis and speciation. Here we review the emerging biology surrounding ferrous iron in bacterial growth and pathogenicity and describe the new chemical biology tools and therapeutic approaches being explored to exploit ferrous iron at the interface of infection and immunity.

Bacterial Iron Homeostasis

Transition metals such as iron, manganese, copper, and zinc, are essential micronutrients for most pathogenic bacteria, with an estimated 30% of proteins in the bacterial proteome using metal cofactors to enable their cellular functions. Bacterial life evolved in a reducing, anoxic environment where reduced forms of iron were prevalent and were apparently co-opted by early life for the redox chemistry they enabled. The 'great oxidation' that coincided with the evolution of photosynthesis presented early bacterial life with an existential challenge, since ferrous iron readily converts oxygen to toxic reactive oxygen species, whilst yielding water insoluble ferric hydroxides. Continued utilization of iron thus required the evolution of iron chaperones (siderophores) that could bind and solubilize the ferric ion, as well as new biochemical pathways to detoxify reactive oxygen species. While the study of bacterial iron homeostasis has historically focused on transport and storage of the ferric ion, there has been increasing study and appreciation over the past decade that bacteria have not lost the means to utilize ferrous iron [9], enabled by biochemistry that is evolutionarily ancient [10].

The 'labile iron pool' (LIP) of a bacterial cell consists of low molecular mass ferrous (Fe^{2+}) iron species bound by still poorly defined cellular ligands (Figure 1). Mössbauer and electron paramagnetic resonance (EPR) spectroscopy of live *E. coli* puts the concentration of LIP in the low-mid micromolar range [11], but LIP levels can be significantly higher than this during exponential-stage growth as detailed below [12]. Bacteria employ various iron regulatory proteins to maintain the LIP and can utilize this system to sense iron depletion as a marker of host tissue during infection [13]. These proteins modulate LIP by activating the expression of metal-acquisition systems under iron limiting conditions or by the expression of efflux transporters and storage proteins under iron replete conditions [14]. The regulation of metal homeostasis involves both transcriptional and post-transcriptional control, as recently reviewed [15]. The Fur (Ferric uptake regulator) family of iron regulatory proteins are ubiquitous in bacteria and regulate the binding of Fe^{2+} (or mismetallation with Mn^{2+} in some instances) from the LIP in order to initiate the transcription of iron-regulatory genes

[16]. Genetic knockouts of the *fur* gene in several bacterial species have shown variable and species-specific effects including decreased virulence [17] and impairment of the growth of bacterial colonies [18]. New players in bacterial iron homeostasis continue to be identified, including a family of Fe^{2+} transporters (IroT/MavN) residing within a specialized vacuole in the bacteria *Legionella pneumophilia* [19].

Metal-sensing regulatory elements called riboswitches have recently been shown to modulate bacterial gene expression in response to different metal ions including Ni^{2+}/Co^{2+} [20], Mn^{2+} [21–23], Mg^{2+} [24] and Fe^{2+} [25]. The riboswitch czcD (NiCO) employed by the human gut microbe *Erysipelotrichaceae bacterium*, was shown to preferentially bind Fe^{2+} (and to a lesser extent Mn^{2+}) under physiological conditions [25]. Previous studies of czcD performed under aerobic conditions had erroneously suggested it was unresponsive to iron, highlighting the importance of considering oxygen tension when studying an oxidation-prone analyte like iron. Studies in the Gram-negative pathogen *Treponema denticola* revealed the presence of a cytoplasmic oxygen-binding di-iron (ODP) metalloprotein that functions as a chemoreceptor to sense both oxygen and ferrous iron for various bacterial signal transduction pathways [26].

Bacteria possess two ferritin-like iron storage proteins, the bacterial ferritin (FtnA) and bacterioferritin (Bfr) as recently reviewed [27]. In *P. aeruginosa* under iron limiting conditions, cytosolic BfrB binds to the bacterioferritin-associated ferredoxin (Bfd) and accepts electrons from its [2Fe-2S] cluster, causing Fe^{3+} stored within BfrB to be reduced and mobilized as Fe^{2+} [28]. The essentiality of the BfrB:Bfd interaction for *P. aeruginosa* has been demonstrated by genetic deletion of the *bfd* gene [28] and with small-molecule inhibitors [29]. However, other recent studies have questioned whether these bacterial proteins serve the same essential iron storage function as eukaryotic ferritins. Using Mössbauer and EPR spectroscopy, Lindahl and co-workers found significant elevation of LIP concentrations (as high as 500 μM!) in *E. coli* during exponential growth and conversely found little spectroscopic evidence for FtnA or Bfr bound iron [12]. Moreover, the major ferric signature observed during stationary growth was present even in FtnA or Bfr deletion strains, and ascribed to Fe^{3+} oxyhydroxide nanoparticles. Based on these findings, it was suggested that a ‘respiratory shield’ comprising membrane-bound enzymes of the electron transfer chain serve to maintain a microaerobic cytoplasm in which even high-μM concentrations of LIP can persist during exponential growth without causing cellular damage. If correct, these findings imply that bacterial cells should be easily distinguishable from eukaryotic cells in their iron speciation and invites strategies to target bacteria on this basis, as will be discussed later.

Ferrous Iron at the Interface of Host-Pathogen Interaction

The concept of “nutritional immunity” [30] – the limitation of systemic iron and other nutrient resources from a pathogen – lies at the heart of the innate immune response as has been well reviewed [9,31,32]. During the mammalian response to bacterial infection, interleukin-6 (IL-6) acts as the main pro-inflammatory cytokine, mediating the release of the peptide hormone hepcidin from the liver. Direct binding of hepcidin to the cellular iron exporter ferroportin then leads to its internalization and degradation, promoting iron

accumulation in phagocytic cells [33]. The expression of mammalian iron-binding proteins such as ferritin, transferrin and lactoferrin also plays a crucial role in restricting the iron resources available to invading pathogens [32]. The host protein lipocalin 2 is another effector of innate immunity, recognizing bacterial siderophore-bound iron (primarily enterobactin) [34]. In addition to withholding of Fe and Mn to limit bacterial proliferation, recent evidence has also suggested that copper and zinc can be weaponized to poison intracellular bacteria via phagocyte-mediated delivery to the phagosome [35–38].

To overcome iron withholding by the host, pathogenic bacteria employ diverse tactics to acquire the iron they need for growth and virulence. Best studied among these strategies is the secretion of Fe^{3+}-chelating small molecules called siderophores (Figure 2a). Upon binding of the ferric ion, a soluble Fe^{3+}-siderophore complex is formed that can be transported into a bacterium and unloaded in either the periplasm or cytoplasm depending on the specific siderophores and transporters involved [39,40]. Recent studies in *P. aeruginosa* have identified complex networks of interacting proteins and transporters that enable, for example, the reduction of ferric iron bound to pyoveridine (PVD, Figure 1). This reduction step takes place in the bacterial periplasm and liberates Fe^{2+} from the PVD-Fe^{3+} complex, where it can bind to chaperone proteins and be shepherded into the cytoplasm [40–43]. Siderophores such as PVD in *P. aeruginosa*, have also been shown to function as signaling molecules in the production of bacterial virulence factors [44,45]. Proteomics experiments investigating the iron starvation response in *P. aeruginosa* have revealed a variety of novel compensatory responses made by the pathogen in order to survive iron-limitation, which includes the upregulation of proteins required for iron-sulfur cluster biogenesis [46].

The host protein calprotectin (CP) is a metal-sequestering protein released from host neutrophils as part of the metal-withholding innate immune response (Figure 2b). While the ability of CP to bind Zn^{2+} and Mn^{2+} has been recognized for some time, work from Nolan and co-workers over the past decade has revealed a high affinity of the His_6 site in CP for Fe^{2+} [47], and this has uncovered a hitherto unappreciated role for CP in limiting Fe^{2+} [48] during host-pathogen interaction, as recently reviewed [49]. The high affinity of the CP His_6 site for the Fe^{2+} ion can even shift the iron redox equilibrium in favor of the ferrous ion under aerobic conditions, an effect that is further facilitated by pyocyanins, redox-cycling phenazines produced by *P. aeruginosa* [50,51]. Further emphasizing the complex and multifaceted role of CP in innate immunity is a recent report [52] that CP can inhibit the growth of *Borrelia burgdorferi*, the pathogen responsible for Lyme disease, by physical interaction with the bacterium, absent metal sequestration.

Under conditions where the Fe^{2+} ion predominates over Fe^{3+} (e.g., in highly acidic, reducing and/or anaerobic conditions), bacteria utilize the ferrous iron transport system FeO (Figure 1) as the primary means to import Fe^{2+} into the cytoplasm [53]. Like PVD siderophores, the FeO system has been identified as a major contributor to the virulence of *P. aeruginosa,* helping the bacterium colonize hypoxic environments [54] such as in the lungs of cystic fibrosis (CF) patients [55,56]. The production of aforementioned pyocyanin metabolites by *P. aeruginosa* is implicated in chronic CF infection where these redox-cycling small molecules are proposed to liberate Fe^{2+} from host proteins in the extracellular environment [57], leading to Fe^{2+} uptake via the transporter FeoB [58]. Phenazine production has also

been reported to promote biofilm formation [55,57] and to increase tolerance to clinically relevant antibiotics [59].

The First Reactivity-Based Probes of Ferrous Iron

Increased appreciation for the role of Fe^{2+} in bacterial growth and innate immunity, as described in the prior sections, has coincided with the development of new chemical probes to study iron with oxidation-stage specificity. While chelation of the electron-poor ferric iron is dominated by σ-donation from electron-rich ligands (e.g., catechol and hydroxamate siderophores), the more electron-rich ferrous ion can engage in back-donation from metal *d*-orbitals to heterocyclic ligands such as the imidazole ring of histidine (e.g., as in the His_6 site in CP). While some measure of selectivity can be achieved in chelation-based iron probes [60], the true breakthrough in oxidation-state specificity has been realized through reactivity-based sensing, as recently reviewed [61]. First generation reactivity-based probes Rho-Nox1 [62] and IP-1 [63] gave early indications that this approach might improve oxidation-state selectivity as compared to chelation-based probes like PhenGreen SK [64]. Further development of the RhoNox-family of probes by Hirayama and co-workers (Figure 3a) has yielded new tools to study Fe^{2+} in the endosome-lysosome system [65], at the plasma membrane [66], in hypoxic tumor spheroids [67], in cellular models of the blood-brain barrier [68] and neural vascular barrier [69], and in mammalian cells undergoing ferroptosis [70], a form of non-apoptotic, iron(II)-dependent cell death [71]. A separate class of reactivity-based probes inspired by the antimalarial trioxolane (TRX) arterolane was described by Renslo and co-workers [72,73] and further developed by Chang [74,75] and Zhang [76] (Figure 3b). The TRX-based probes TRX-PURO [72] and FIP-1 [74] were able to detect an increase in LIP in cancer cell lines and revealed a previously unappreciated link between GPCR signaling and epigenetic regulation by mononuclear Fe^{2+}-dependent TET enzymes [77,78].

These new tools have only just begun to be applied to the study of Fe^{2+} in bacterial iron-homeostasis and in bacterial infection. Thus, after first establishing that ICL-1 (Figure 3c) was highly selective for reaction with the Fe^{2+} ion over other biologically relevant metals and reductants, this caged form of luciferin was used to detect the mobilization of Fe^{2+} in luciferin-expressing mice (FVB-luc+) infected with the Gram-negative pathogen *A. baumannii* [75]. Whereas ICL-1 treatment of mock-infected control animals showed only weak bioluminescence near the intra-peritoneal site of ICL-1 administration, infected mice treated administered ICL-1 showed more dramatic bioluminescence in organs that were later shown by *ex vivo* analysis to also be major sites of infection (Figure 3d). Analysis of total iron load by ICP-MS in the infected tissues showed a substantial elevation of total iron only in liver, an observation consistent with canonical nutritional immunity and iron sequestration by that organ. The bioluminescent signal in the other infected tissues was not correlated with total iron load however, and thus indicates that Fe^{2+} specifically is elevated during infection. This mobilization of Fe^{2+} might reflect LIP (Fe^{2+}) expansion by a pathogen undergoing exponential growth, as described by Lindhal in the spectroscopic studies described above [12]. Alternatively, these changes might reflect the pathogen's utilization of extracellular Fe^{2+} in the infection microenvironment [53,55], or the canonical unloading of Fe^{2+} from siderophore–Fe^{3+} within the pathogen. Further studies will be required to address the

questions posed by these recent studies and new tools, which are challenging some long-held assumptions about iron utilization and sequestration during infection.

Antibiotic therapy exploiting iron has been well explored for siderophore-antibiotic conjugates ('sideromycins') designed to undergo active uptake via bacterial Fe^{3+}-siderophore transporters [79,80]. More recent findings concerning Fe^{2+} in infection, as detailed herein, invite study of a new therapeutic approach comprising the reactivity-based activation of antibiotics selectively at infection sites, in response to Fe^{2+}. To explore this notion, our laboratory recently described [81] the design and synthesis of an Fe^{2+}-activatable form of the LpxC inhibitor PF-5081090 (i.e., TRX-PF508) and its study in *P. aeruginosa* infection models (Figure 3e). Whereas our previously described Fe^{2+} sensors [73,82] were designed to cage amine-bearing payloads, the TRX sensor employed in TRX-PF508 enables caging of hydroxamate-based metalloenzyme inhibitors, potentially mitigating the well-known [83] toxicological and pharmacokinetic liabilities of the hydroxamate function. Intriguingly, whereas TRX-PF508 showed only weak activity in MIC experiments, the compound was highly efficacious in an acute *P. aeruginosa* lung infection model, reducing bacterial CFU counts in a dose-dependent fashion and being well tolerated even at the highest dose of 64 mg/kg. By contrast, mice administered the parent drug PF-5081090 showed only a modest (non-significant) reduction in lung CFU counts at a dose equimolar to the lowest effective dose of TRX-PF508 (16 mg/kg). Overall, the findings of this study were consistent with a drug concentrating effect of TRX-PF508 in the lung, presumably resulting from selective activation by Fe^{2+} in this (infected) tissue.

Here we have summarized recent studies of bacterial acquisition and utilization of ferrous iron during infection and in the context of host-pathogen interaction. New chemical probes of ferrous iron emerging contemporaneously with these microbiological studies invite future opportunities to apply these new tools to advance a more nuanced understanding or iron acquisition and specification, both *in vitro* and *in vivo*. Early studies of this type include the imaging of Fe^{2+} in a murine infection model with ICL-1 and the development of antibiotics conjugates like TRX-PF508 that are activated in the infection microenvironment by reactivity-based sensing of Fe^{2+}. These new tools and emerging biology suggest a bright future for the field of chemical (micro)biology.

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* of special interest

** of outstanding interest

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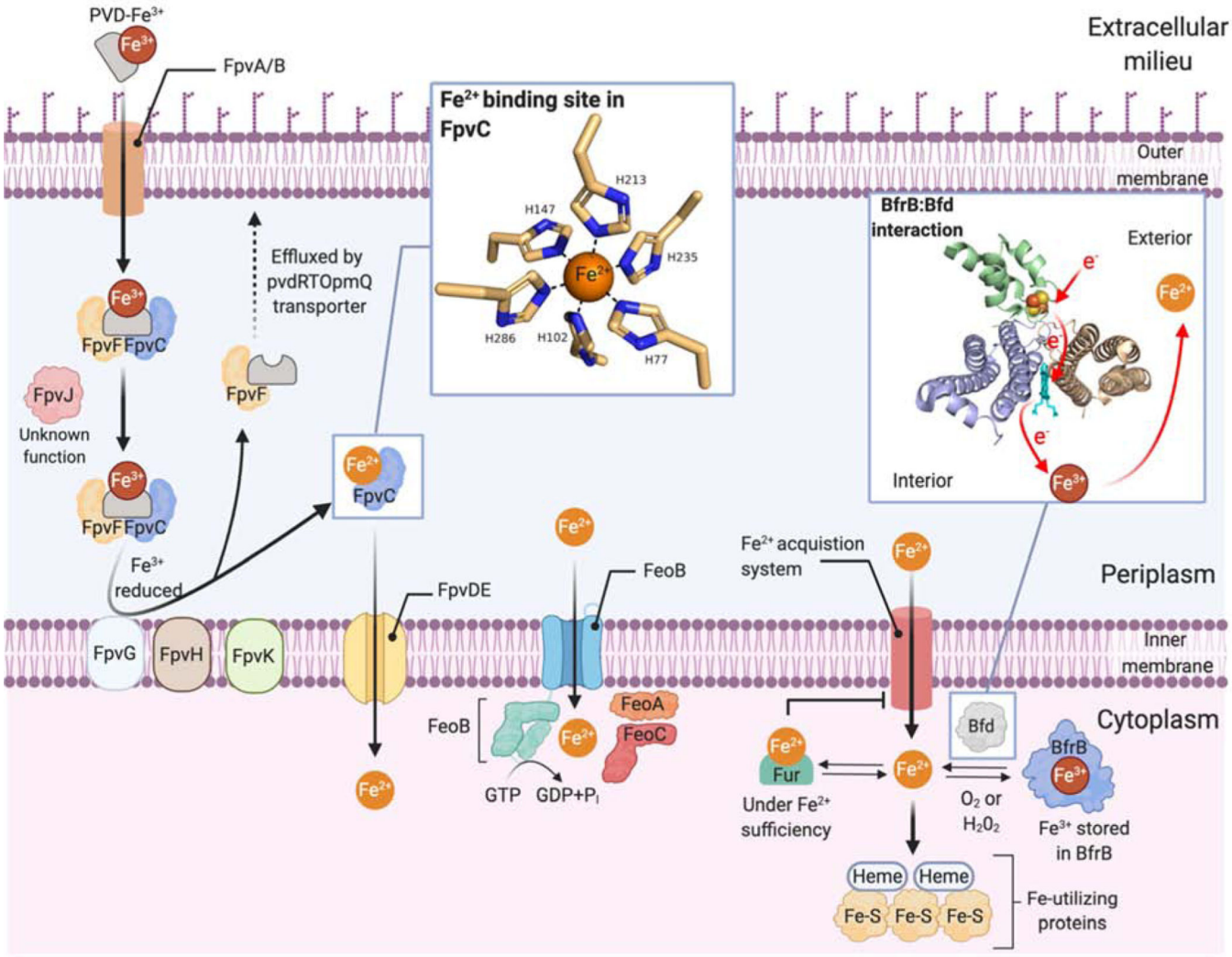

Figure 1.

Overview of iron acquisition, storage, and transport in Gram-negative bacteria with an emphasis on mechanisms of ferrous iron (Fe^{2+}) uptake and mobilization from ferric (Fe^{3+}) forms. The figure summarizes recent findings from studies conducted in more than one species of Gram-negative bacteria.

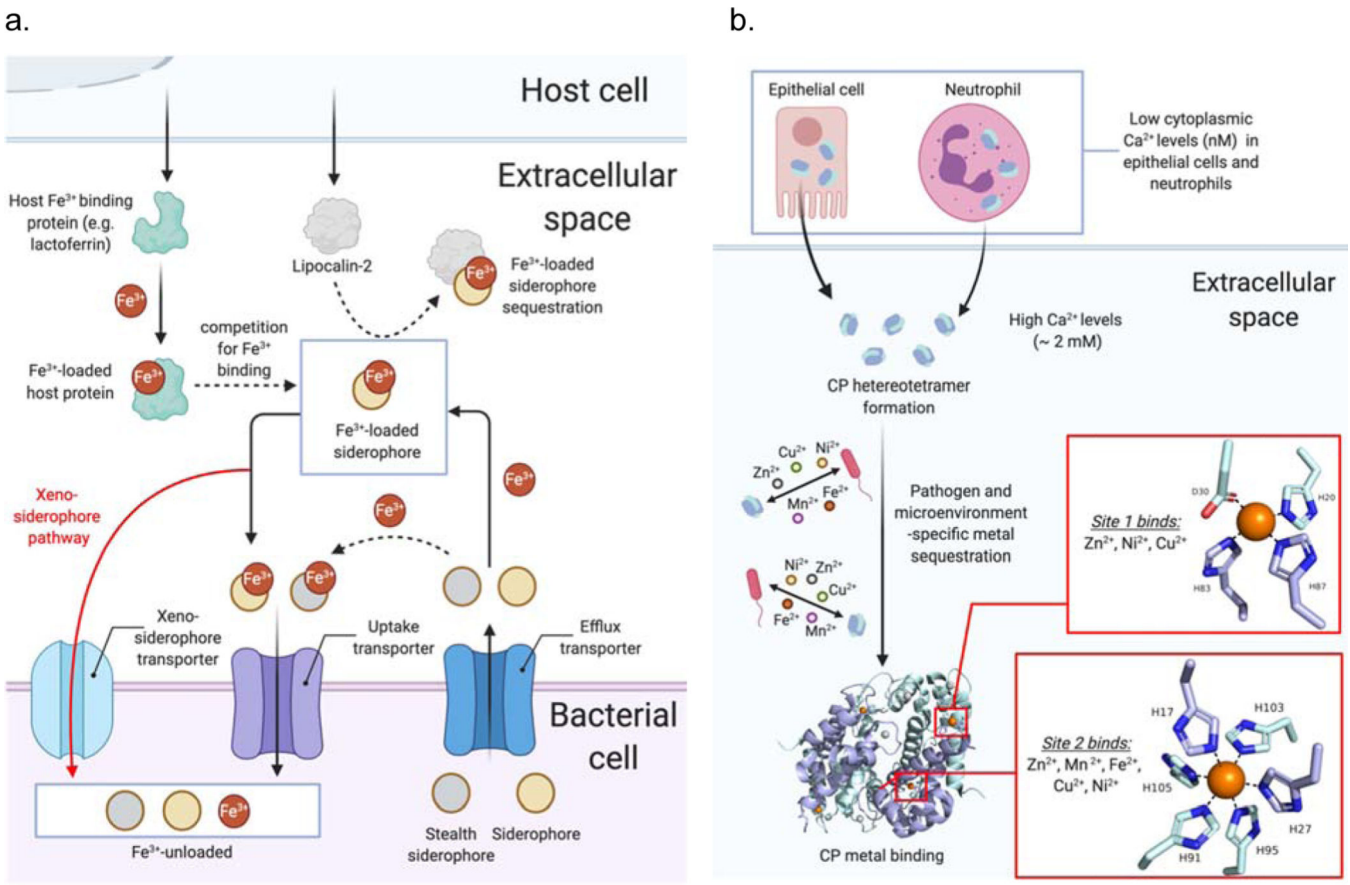

Figure 2.

Iron and other metal ions during host-pathogen interaction. (a) Competition for Fe^{3+} resources is centered around bacterial siderophores and mammalian Fe^{3+} and siderophore-binding proteins. (b) Calprotectin (CP) is a general metal sequestering protein the binds Fe^{2+} and other divalent metal ions in the extracellular environment.

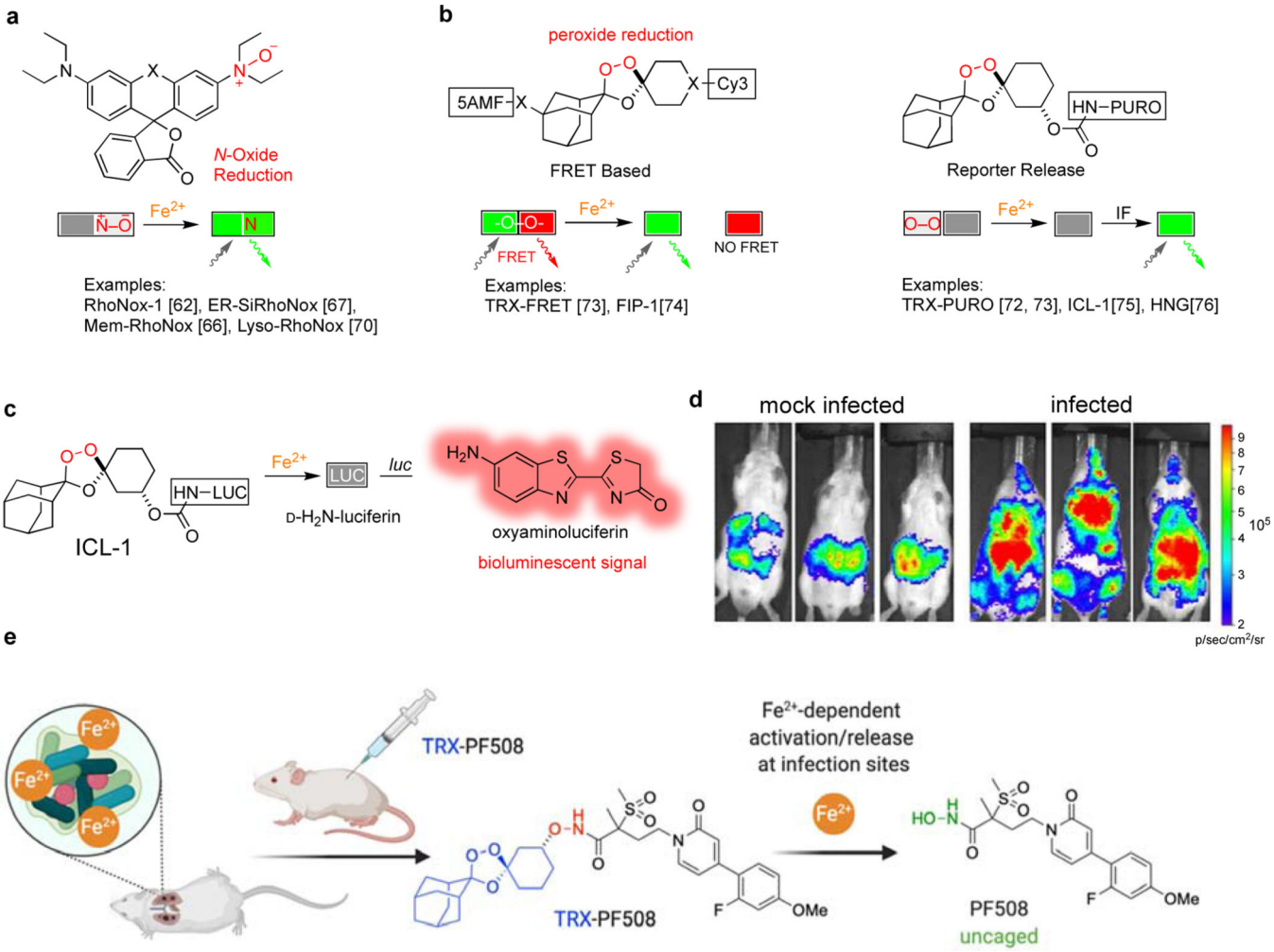

Figure 3.

New tools to study ferrous iron *in vitro* and *in vivo*. (a) RhoNox-family probes are activated by reduction of an *N*-oxide bond; (b) TRX-based probes are activated by reduction of a hindered peroxide bond; (c) The probe ICL-1 releases D-aminoluciferin and can be used in luciferin-expressing cells and animals; (d) mice infected with *A. baumannii* show elevated Fe^{2+} as detected with ICL-1; (e) the drug conjugate TRX-PF508 releases an LpxC inhibitor upon sensing Fe^{2+} *in vivo* and is efficacious in an acute *P. aeruginosa* lung infection model.