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Siderophore-mediated iron acquisition and modulation of host-bacterial interactions

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Abstract

Iron is an essential micronutrient for most life forms including the majority of resident bacteria of the microbiota and their mammalian hosts. Bacteria have evolved numerous mechanisms to competitively acquire iron within host environments, such as the secretion of small molecules known as siderophores that can solubilize iron for bacterial use. However, siderophore biosynthesis and acquisition is not a capability equally harbored by all resident bacteria. Moreover, the structural diversity of siderophores creates variability in the susceptibility to host mechanisms that serve to counteract siderophore-mediated iron acquisition and limit bacterial growth. As a result, the differential capabilities to acquire iron among members of a complex microbial community carry important implications for the growth and function of resident bacteria. Siderophores can also directly influence host function by modulating cellular iron homeostasis, further providing a mechanism by which resident bacteria may influence their local environment at the host-microbial interface. This review will explore the putative mechanisms by which siderophore production by resident bacteria in the intestines may influence microbial community dynamics and host-bacterial interactions with important implications for pathogen- and microbiota-driven diseases including infection, inflammatory bowel diseases and colorectal cancer.

Graphical abstract

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Conflict of interest

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Keywords

Intestinal microbiota; iron homeostasis; siderophore; host-microbial interactions; infection; inflammation

Introduction

The gastrointestinal (GI) tract is home to a collection of microbial communities collectively known as the intestinal microbiota. In fact, nearly all other healthy body sites that are colonized with distinct microbial communities comprise a specific host-associated microbiota [1] [2]. At birth, the GI tract becomes rapidly colonized with a community of microbes that over the first several years of life, increases in complexity and stabilizes into a mature state [3] [4]. The bacterial phyla Firmicutes and Bacteroidetes comprise the majority of the normal adult enteric microbiota, with Actinobacteria, Proteobacteria and Verrucomicrobia present in lesser abundances [5]. However, the composition of the microbiota is not static and undergoes temporal variations as a result of environmental factors including but not limited to dietary changes, exposure to pathogens and xenobiotics, inflammation and overall health status of the host [6] [7] [8] [9] [10] [11]. Moreover, variations in the spatial distribution of nutrient availability and host-derived factors results in compositionally and metabolically distinct bacterial communities residing within the lumen, at the mucosa, and longitudinally along the GI tract [12] [5] [13] [14].

The intestinal microbiota, in symbiosis with the host, is integral to numerous host processes including immune system development and nutrient metabolism [15] [16] [17] [18] [19] [20] [21]. The intestinal microbiota is physically separated from the underlying mucosal immune system by a single layer of epithelial cells, a thick layer of mucus and host secretions including antimicrobial peptides and soluble immunoglobulin A (IgA) antibodies that collectively make up the intestinal barrier. The mucosal immune system is tasked with remaining tolerant of resident microbes while responding to pathogens and other microbes that breach the intestinal barrier in order to limit uncontrolled inflammatory responses and systemic dissemination [22]. However, resident bacteria do not play a passive role in maintaining this symbiotic relationship. Depletion of specific bacterial groups through the use of antibiotics and colonization of germ free mice with a single bacterial strain or defined community (i.e. gnotobiotic mice) have revealed that select members of the intestinal

microbiota can modulate specific host responses within the intestines [23]. Some resident bacteria induce tolerogenic and anti-inflammatory immune responses and enhance barrier function [24] [25] [26] [27] [28], while others favor the establishment of a more proinflammatory microenvironment [25] [29] [30]. Blooms of resident bacteria with greater proinflammatory potential and/or their mislocalization to mucosal sites enhance the opportunity for inappropriate microbial stimulation of the mucosal immune system, which can ultimately compromise the symbiotic relationship between the microbiota and the host. These unfavorable compositional and functional changes to the microbiota, collectively known as dysbiosis, have been associated with acute inflammatory insults such as enteric infection as well as chronic diseases including inflammatory bowel diseases (IBD) and colorectal cancer [31] [32] [33] [34] [35].

Nearly all bacteria and their mammalian hosts compete for iron, an essential micronutrient. Iron serves as a cofactor for numerous cellular proteins involved in diverse processes including DNA synthesis and repair, cellular respiration, biodegradation and biosynthetic pathways and transcriptional regulation [36]. However, given the propensity for free iron to participate in Fenton chemistry and generate toxic reactive oxygen species (ROS), the abundance of free iron must be tightly regulated. Under most physiological conditions (i.e. oxygenated and neutral pH), non-heme iron predominates in its ferric form and exhibits low bioavailability. To overcome this, both host and microbe encode enzymes that reduce ferric iron into its more soluble ferrous form and secrete proteins or small compounds termed siderophores that sequester extracellular ferric iron for cellular use. The necessity of iron for growth of most pathogenic invaders and resident bacteria sets the stage for an evolutionary battle for iron between host and microbe at the mucosal interface. Indeed, as a key component of the host innate immune response, bacterial iron availability is limited by mucosal secretion of host iron binding proteins to prevent systemic dissemination of pathogens and ensure luminal compartmentalization of endogenous bacteria to maintain symbiosis. However, some bacteria taxa – both pathogen and resident – have acquired mechanisms of iron acquisition that overcome attempts by the host to limit iron availability, a competitive advantage that can impact interbacterial interactions within the microbiota and interkingdom interactions between resident bacteria and their hosts [37] [38] [39] [40]. This review will largely focus on how siderophore-mediated bacterial iron acquisition can shape bacterial community dynamics and host-microbial interactions within the intestinal environment.

Siderophore-mediated iron acquisition

Bacterial iron homeostasis is tightly regulated to enable the acquisition of sufficient iron while also limiting toxicity from iron-mediated production of reactive oxygen species. Accordingly, the expression of iron acquisition systems is restricted to environments where iron stores are depleted. Changes in intracellular iron concentrations are sensed by the ferric uptake regulator (Fur), the master transcription factor of iron homeostasis in a wide range of bacterial taxa [41] [42] [39]. When intracellular iron is replete, the Fur-Fe₂₊ complex inhibits transcription of genes involved in iron acquisition, thus preventing excess iron import into the cell [43] [44]. Under iron limiting conditions, apo-Fur predominates in the

cell, resulting in the de-repression of iron acquisition genes and consequent iron transport into the cell.

Mechanisms of bacterial iron acquisition include siderophore-mediated transport, direct import through divalent metal transporters or direct piracy from iron-bound host proteins [41] [45] [37] [39]. Siderophores are low molecular weight compounds with high affinity for ferric iron, and are synthesized and secreted by bacteria in order to scavenge iron when availability is limited. In Gram-negative bacteria, siderophore-bound iron is transported through cognate outer membrane receptors that require energy transduction by the TonB-ExbB-ExbD protein complex [46] [41]. In the periplasm, a chaperone protein binds the iron-chelate and delivers the complex to cognate ABC permeases on the inner membrane. Gram-positive bacteria utilize a similar mechanism of siderophore transport through ATP-binding cassette (ABC) transporters localized at the cytoplasmic membrane [47]. Once within the cytoplasm, iron is liberated from the siderophore by one of two mechanisms [37]. The more common approach occurs by reduction of ferric iron to its ferrous form by non-specific ferrisiderophore reductases, often flavin reductases that also serve other cellular functions. The decreased affinity for ferrous iron is then thought to enable the spontaneous release of iron from the siderophore. The second mechanism depends on specific enzymatic hydrolysis of the siderophore, which serves to weaken its interactions with iron and enable its liberation. In both Gram-negative and -positive organisms, ferrous iron is directly transported through cytoplasmic membrane permeases or ABC transporters [48] [49]. Some bacterial pathogens are also capable of utilizing host sources of iron by expressing outer membrane receptors that directly bind to host iron binding proteins such as transferrin [45].

Siderophores are a structurally diverse group of compounds that impart distinct functional attributes to bacteria. Siderophores can be categorized into three main structural families – carboxylate, catecholate, and hydroxamate – named accordingly to the functional groups that confer their binding affinity and selectivity for ferric iron [40] [37] [50]. Some common siderophores produced by enteric bacteria are listed in Table 1. There are also many siderophores such as yersiniabactin and aerobactin that either incorporate more than one of these functional groups within their structures or contain additional functional groups that interact with the ferric iron ion. Carboxylate siderophores such as staphyloferrin A exhibit a greater affinity for ferric iron at acidic pH ranges and therefore likely contribute to enhanced fitness within more acidic environments. In contrast, under physiologic conditions, catecholate siderophores such as enterobactin and salmochelin exhibit higher affinity for ferric iron relative to carboxylate or hydroxamate siderophores. Indeed, enterobactin exhibits the highest known affinity for ferric iron and outcompetes the host iron binding protein transferrin for iron, thus enabling bacteria to thrive within transferrin-rich environments [51] [40]. Given the chemical diversity of siderophores, harboring distinct siderophore systems likely imparts unique fitness advantages to bacteria within a varied range of environments. Indeed, encoding numerous siderophore systems is associated with enhanced fitness for both pathogenic and resident bacteria within the intestines [52] and at many extraintestinal sites [53] [54] [55] [51].

Bacterial iron availability in the GI tract

Within the GI tract, a major source of iron for resident bacteria comes from the diet. About 5–15% of non-heme iron is absorbed in the duodenum [56], leaving the remaining unabsorbed iron to pass through the intestines. Supporting the role of diet as a modulator of colonic iron concentrations, consumption of an iron-fortified diet or oral iron supplements increases total non-heme iron concentrations in fecal contents (Table 2) [57] [58] [59] [52] [14] [60] [61]. However, measurements of total iron concentrations may not accurately reflect actual bioavailability of iron for resident bacteria, as this measurement includes bacterial intracellular iron stores and chemically inaccessible forms of iron. Interestingly, in comparison to conventionalized mice (i.e. former germ free animals that have been colonized, such as via fecal transplantation), total cecal iron concentrations are 10-fold higher in germ free mice, while systemic parameters of host iron status remain unchanged [60]. Liver transcript levels of the hormone hepcidin, which limits duodenal iron absorption when increased, were also unaltered. Colonization of germ free mice with representatives of two major phyla of the microbiota, the resident Bacteroidetes bacteria *Bacteroides thetaiotamicron* and Firmicutes bacteria *Faecalibacterium prausnitzii*, did not decrease cecal iron levels [60], suggesting that other specific resident bacteria modulate total iron concentrations through some undefined mechanism.

Free and chelatable iron, together known as readily exchangeable iron, can also be quantified in fecal samples. Free iron is a measure of the concentration of soluble ferric and ferrous iron in fecal water, while chelatable iron is quantified by measuring iron in fecal water after treatment with the metal chelator ethylenediaminetetraacetic acid (EDTA) [57] [58]. Together, readily exchangeable iron may more appropriately reflect bacterial iron availability, as it can directly participate in Fenton chemistry and presumably be directly utilized by bacteria. Approximately 1–5% of total fecal iron in humans is comprised of readily exchangeable iron [57] (Table 2). As with total fecal iron, concentrations of readily exchangeable iron are also sensitive to dietary changes (Table 2).

Gradients of iron availability exist across the GI tract, where maximal concentrations can be found within the lumen and decrease towards the mucosa [14]. Host processes that occur during steady-state conditions, together with the biogeography of the intestines, contribute to the establishment of these varying pockets of iron availability. For example, homeostatic processes such as the cyclic shedding of iron-laden enterocytes likely contribute to increasing luminal iron concentrations. In contrast, as part of the innate immune system, mucosal secretions of iron binding proteins such as lipocalin-2 (Lcn2) and lactoferrin serve to decrease microbial iron availability at the mucosa [62] [63] [64]. Indeed, in the colon, total iron concentrations were reported to be approximately 4–5 fold lower within the mucus layer compared to luminal contents [14]. The increased oxygenation of the mucosal environment may also further diminish the bioavailability of iron as this favors the presence of the more insoluble ferric iron species.

Host inflammation status has also been shown to modulate intestinal iron availability. In a *Salmonella*-induced colitis model, total fecal iron levels were decreased compared to mock-infected controls [52]. Interestingly, this was associated with significant weight loss, which

in the context of unaltered hepcidin levels, points to decreased appetite as a possible mechanism for decreased fecal iron. Similarly, inflammation enhances mucosal secretion of host iron binding proteins in *Salmonella*-induced colitis, in the interleukin 10 (*Il10*)^{-/-} chronic colitis model and in IBD patients, which also limits bacterial iron availability [65] [63] [66] [67]. Decreased absorption of dietary iron as a result of inflammation-mediated upregulation of hepcidin may further increase luminal iron concentrations [68]. Indeed, development of intestinal inflammation has been associated with increased liver expression of hepcidin and corresponds with decreased systemic iron stores in several rodent models of colitis [69] [70] [71]. Thus, inflammation can play divergent roles in modulating intestinal iron concentrations. Taken together, although the precise bioavailability of iron for resident bacteria remains unclear, dietary and host factors both contribute to the modulation of microbial iron availability within the intestines.

Evidence for siderophore production in the intestines

Direct detection or quantification of siderophores in most types of intestinal samples, such as feces or mucosal tissues, is a technically challenging feat given the complexity of chemically diverse metabolites present within these samples. Nonetheless, in a recent study, Pi and colleagues measured enterobactin from the cecal mucus of conventionally raised mice [72]. In the same study, administration of streptomycin, an antibiotic that targets aerotolerant bacteria including Enterobacteriaceae, resulted in a 2-fold decrease in enterobactin concentrations, suggesting that endogenous Enterobacteriaceae such as *E. coli* are a source of mucosal enterobactin. *E. coli* production of enterobactin has also been demonstrated *ex vivo* when cultured in mucus isolated from germ free mice and corresponds with an *in vivo* upregulation of enterobactin biosynthesis and uptake genes in the mucus versus luminal environment [14]. Taken together, these studies provide strong biochemical evidence that enterobactin biosynthesis occurs within the intestinal environment.

To our knowledge, the detection of other bacteria-derived siderophores in the intestines has not been reported. Nonetheless, bacterial genetic studies have provided ample evidence that resident and pathogenic Enterobacteriaceae express siderophore systems upon colonization of the intestinal environment. For example, an aerobactin producing *E. coli* strain outcompetes a transport mutant lacking the aerobactin receptor IutA within cecal contents and tissues [73], suggesting a role for this siderophore system in promoting optimal growth within the cecal environment. Similarly, a *S. enterica* transport mutant lacking the salmochelin receptor IroN exhibits a competitive disadvantage in the intestinal lumen [63]. With the exception of enterobactin, we are unaware of similar studies investigating the contribution of a single siderophore system to the intestinal fitness of resident intestinal isolates. However, simultaneously inactivating all siderophore- and heme-mediated iron transport decreases fecal colonization of an intestinal *E. coli* isolate when grown in competition with *S. enterica* [52].

Siderophore production is partially regulated via transcriptional control of genes encoding its biosynthetic machinery, and there is evidence of *in vivo* transcriptional regulation of these siderophore systems in several bacterial strains. In fact, *in vivo* transcriptional regulation of siderophore systems has been found both in pathogenic and resident intestinal

Enterobacteriaceae. Bacterial RNA-seq revealed temporal modulation of genes involved in siderophore biosynthesis and transport from the fecal environment of mice colonized with resident intestinal *E. coli* NC101, a strain that encodes enterobactin, salmochelin and yersiniabactin [74]. Similarly, when localized to the Peyer's patches, *Yersinia enterocolitica* is positive for expression of the yersiniabactin receptor FyuA, albeit at much lower levels compared to extraintestinal sites such as the peritoneal cavity [75]. From an evolutionary perspective, it is interesting to note that fecal *E. coli* isolates, compared to environmental isolates, more frequently harbor yersiniabactin and aerobactin genes and generally encode a higher number of siderophore systems [76]. Similarly, yersiniabactin and aerobactin genes are present in a larger percentage of human enteric *E. coli* isolates of the B2 and D phylotypes [77] [78], which together comprise the majority of intestinal resident *E. coli* [79]. Taken together, these findings provide strong evidence that siderophore biosynthesis occurs within the intestinal environment and likely contributes to niche maintenance and fitness. Defining which resident bacteria of the microbiota secrete and utilize siderophores to acquire iron in the intestines remains an open question worth exploring.

Siderophores as modulators of microbial communities

Iron availability is an environmental factor that impacts the composition and function of many microbial communities. This comes as no surprise given the requirement of iron as a growth factor for most bacteria and the differential capacities to scavenge for this micronutrient among distinct bacterial taxa. In the colon, changes in iron concentrations through dietary interventions correspond with compositional changes to the intestinal microbiota as well as significant shifts to the fecal metabolome [80] [59] [81] [82] [83] [84] [85] [61]. Manipulation of iron availability also compositionally and functionally alters fecal microbial communities in an *in vitro* colonic fermentation model [86] [87], demonstrating that iron can modulate microbial communities in the absence of host intrinsic factors. Because siderophores bind up iron and restrict its availability to bacteria that express their cognate siderophore receptors, it is conceivable that siderophores could also modulate local iron availability and impact community dynamics by modulating bacterial growth, physiology and metabolic activities and consequent bacterial interactions with the host. Possible mechanisms by which this may occur are explored in this section and are summarized in Figure 1.

Interbacterial competition

The iron requirements necessary for optimal growth and strategies used to competitively acquire this micronutrient vary among members of the intestinal microbiota. The majority of taxa present within intestinal communities remain poorly characterized and thus little is known about the impact of iron on their growth and function or their mechanisms of iron acquisition. Nonetheless, studies that have characterized the compositional changes that occur as iron availability is altered provide some clues as to which members of the fecal microbiota are responsive to fluctuations in iron and which exhibit a competitive advantage when availability is decreased.

Iron-dependent bacteria competitively employ different means to acquire iron. Siderophilic bacteria (i.e. capable of utilizing siderophore-bound iron) utilize aggressive means of scavenging iron that enable them to steal iron from host-derived sources while also further restricting local iron availability for their non-siderophilic counterparts. This concept is exemplified through intestinal growth competitions between a non-pathogenic intestinal *E. coli* strain and either a mutant unable to synthesize enterobactin or a mutant unable to transport iron sequestered by enterobactin and other catecholate siderophores [72]. These studies revealed a more dramatic growth disadvantage with the transport mutant, which like non-siderophilic bacteria, are unable to utilize siderophore-bound iron as an accessible source of iron. In contrast, the enterobactin-negative mutant exhibited a modest decrease in intestinal growth, possibly because catecholate siderophore transport remained intact in this mutant. Indeed, not all siderophilic bacteria produce siderophores for which they express the cognate receptor [41] [30], providing the opportunity for siderophore-negative but receptor-positive bacteria to steal siderophore-bound iron produced by other members of the community.

At the community level, decreased iron availability has been associated with the outgrowth of siderophore-producing bacterial families and reduced abundances of other non-siderophilic iron-dependent taxa within the intestinal microbiota [59] [88] [81] [86] [82] [83] [89] (Ellermann, unpublished). For example, decreased dietary iron in both rodent models and clinical studies corresponds with relative increases in Bifidobacteriaceae including *Bifidobacterium* [59] [82] [83], which were recently shown to be positive for *in vitro* siderophore production [89]. Similarly, iron chelation promotes the outgrowth of Bifidobacteriaceae in colonic fermentators inoculated with a human fecal microbial community, demonstrating that this particular compositional change occurs in the absence of iron-mediated effects on host biology [86] [87] [90]. However, it remains unclear whether siderophore production is increased as intestinal iron availability is diminished as a result of dietary interventions or host intrinsic factors such as inflammation. Nonetheless, one study reported an increased presence of metabolites with potential siderophore activity in response to low iron in an *in vitro* fermentator model [90]. This was associated with an increased abundance of siderophilic *Bifidobacterium* but not Enterobacteriaceae. Similarly, using the metagenomic predictive algorithm PICRUST, the presence of genes involved in siderophore biosynthesis and transport was increased in mice administered an iron deficient diet relative to a control or iron supplemented diet and corresponded with an increased abundance of Enterobacteriaceae (Ellermann, unpublished). Together, these observations support the idea that diminished iron availability may promote siderophore production by certain members of the microbiota and may provide a competitive advantage for siderophiles within the community. Thus it will be interesting to determine how dietary and host factors impact siderophore biosynthesis and secretion through metagenomic, metabolomic and metatranscriptomic studies. Moreover, bacterial genetic studies will be essential to determine the contribution of siderophores to the intestinal growth of siderophilic bacteria and, more broadly, their immunomodulatory effects on the host and ecological effects on the microbial community as a whole.

In contrast, decreased iron availability has been associated with a reduced abundance of iron-dependent taxa that utilize alternative mechanisms to acquire this micronutrient. For

example, several rodent and clinical studies as well as studies utilizing colonic fermentators demonstrated a reduction in *Bacteroides* and *Roseburia* that, to our knowledge, have not been reported to produce siderophores [91] [92] [93] [59] [81] [83] [87] [61]. Interestingly, low iron availability frequently corresponds with significant decreases in fecal concentrations of propionate and butyrate, short chain fatty acids (SCFA) that are metabolic byproducts of microbial anaerobic fermentation [81] [86] [85] [87] [61] [90]. Consistent with these *in vivo* observations, *in vitro* studies with the robust butyrate producer *Roseburia intestinalis* demonstrated that the transcription of genes involved butyrate biosynthesis is iron responsive and that growth in iron-depleted conditions corresponds with a metabolic shift away from butyrate production [87]. This has important implications for host-microbial interactions given that SCFAs such as butyrate serve as an important carbon source for colonic enterocytes and can modulate host immunity and physiology through histone modification and engagement of G-protein coupled receptors [94]. Indeed, SCFAs such as butyrate and propionate exhibit potent anti-inflammatory and anti-carcinogenic properties [94] [95]. Thus it would be interesting to explore how interspecific interactions between siderophore producers and anaerobic fermentative bacteria impacts SCFA production and consequent effects on the host. Taken together, these microbiota studies collectively demonstrate that as intestinal iron availability becomes more restricted, this favors the outgrowth of siderophilic bacteria at the expense of other iron dependent bacterial taxa that play an important role in modulating local host responses.

Local bacterial niche formation

The GI tract can be viewed as a heterogeneous collection of microbial niches, where factors that influence bacterial growth including availability of nutrients vary dramatically throughout the intestines. Because the source (i.e. host versus dietary) and chemical forms of iron differ throughout the intestines, encoding distinct ways to acquire iron may confer resident bacteria the ability to thrive within distinct intestinal microenvironments. Consistent with this idea, maximal production of specific siderophores depends on environmental factors including pH, oxygenation and carbon source [96]. Thus, harboring a repertoire of siderophore systems with unique structural characteristics likely enhances the adaptability of resident and pathogenic bacteria to changing environmental conditions and contributes to bacterial niche selection within the intestines.

Siderophore-mediated iron transport is likely an important fitness factor at the mucosa because physiological conditions there favor the predominance of ferric iron. This is exemplified by the expression profile of iron acquisition genes in mucus-associated versus luminal *E. coli*, where genes involved in enterobactin biosynthesis and transport were uniquely upregulated in the mucus [14]. In contrast, genes involved in ferrous iron transport were not differentially expressed between the two niches, suggesting that the mucus environment favors the expression of siderophore systems. This corresponded with increased rates of proliferation of *E. coli* localized to the mucus compared to the lumen. Whether enterobactin synthesis and transport specifically enhances growth of *E. coli* within the mucus has not been explored. However, in another study, inactivation of siderophore-mediated transport in *S. enterica* significantly decreased its growth within the Peyer's patches while minimally impacting luminal growth [97]. In contrast, a *S. enterica* mutant

unable to directly import ferrous iron through the divalent iron permease FeoB exhibited a more sustained growth defect in the lumen compared to the siderophore transport mutant. Therefore, these studies support the idea that siderophore-mediated iron transport is important for maximizing bacterial fitness at the mucosa.

The innate immune system limits bacterial replication and systemic dissemination in part by modulating the nutrient landscape (i.e. nutritional immunity). To limit the range of accessible iron sources for siderophilic resident bacteria at the mucosal interface, neutrophils and epithelial cells secrete the antimicrobial peptide Lcn2 that binds enterobactin to prevent its bacterial use [98] [62]. Several studies have recently demonstrated the importance of Lcn2 in limiting enterobactin availability. The absence of Lcn2 increased translocation of resident intestinal bacteria to the draining lymph nodes and exacerbated colitis and tumorigenesis in inflammation-susceptible *II10*^{-/-} mice [30]. This change in nutritional immunity was associated with a bloom of *Alistipes* spp. within the fecal microbiota. Interestingly, *in vitro* growth of *Alistipes* was enhanced in the presence of enterobactin and inhibited by Lcn2. Thus Lcn2 appears to restrict the outgrowth of *Alistipes* spp. in part by limiting its access to enterobactin-bound iron. Colonization with *Alistipes* also exacerbated colitis and induced tumorigenesis in Lcn2-sufficient *II10*^{-/-} mice, suggesting that the outgrowth of this particular bacterium promotes inflammation and carcinogenesis in genetically susceptible hosts and may be a feature of a dysbiotic microbiota. Similarly, in the lung mucosal environment, the absence of Lcn2 promoted the mislocalization of enterobactin-producing *K. pneumonia* from the airways to the transferrin-rich environment of the perivasculature [51]. This corresponded with an unfavorable redistribution in the pattern of histological inflammation from the airways to the perivasculature. Taken together, these findings demonstrate the importance of nutritional immunity and host Lcn2 in restricting the replicative niche of siderophilic bacteria that utilize enterobactin-bound iron. Moreover, Lcn2-mediated modulation of bacterial iron availability serves as a key innate immune mechanism for maintaining symbiosis and limiting bacterial-induced inflammation.

To counteract mucosal nutritional immunity, many bacteria synthesize additional siderophores that are resistant to Lcn2 binding. Some Enterobacteriaceae secrete salmochelin, a form of enterobactin that is glycosylated, which prevents its recognition by Lcn2 [99]. The secretion of non-catecholate siderophores such as yersiniabactin and aerobactin also confers microbial resistance to Lcn2 [50] [40]. The ability to import Lcn2-resistant siderophores increases the competitive advantage of intestinal resident and pathogenic Enterobacteriaceae in the inflamed intestines and urinary tract, growth advantages that are lost in Lcn2-deficient mice [63] [52] [67] [100]. Similarly, in a lung infection model, the absence of Lcn2 did not alter the replicative niche of yersiniabactin-producing *K. pneumonia* [51]. Thus, the ability to utilize Lcn2-resistant siderophores counteracts attempts by the host to limit bacterial niche formation within mucosal environments. Interestingly, the yersiniabactin siderophore system is overrepresented in the genomes of resident *E. coli* isolates recovered more frequently from Crohn's disease patients compared to healthy controls [101] [78]. These resident *E. coli* strains exhibit unique functional characteristics, such as increased epithelial invasiveness and the ability to induce colitis in numerous rodent models [102] [103] [104] [105] [106] [107], and may therefore contribute to the maintenance of a proinflammatory intestinal environment in Crohn's

disease. Thus features of the inflamed environment, such as elevated Lcn2 secretion, may provide selection pressure for the retention of members of the intestinal microbiota that harbor Lcn2-resistant siderophore systems.

The expression of siderophore systems is not always detrimental to host health. In fact, siderophore-mediated iron acquisition by probiotic or beneficial microbes promotes colonization resistance towards invading intestinal pathogens. For example, colonizing *S. enterica*-infected mice with the probiotic *E. coli* strain Nissle reduced fecal burdens of *S. enterica* and ameliorated histopathology and host proinflammatory responses [52]. Inactivating all TonB-dependent iron transport in *E. coli* Nissle, which effectively disrupts the enterobactin, yersiniabactin, salmochelin and aerobactin siderophore systems, abrogated its ability to outcompete *S. enterica*. Thus it will be interesting to determine how distinct siderophore systems harbored by resident *E. coli* may contribute to resistance against enteric pathogens.

In addition to the siderophore repertoire harbored by a particular bacterial strain, the relative amounts of various siderophores secreted by a population of bacteria may also contribute to intestinal fitness and local niche formation. Under identical growth conditions, strains that encode the same functional siderophore systems secrete different proportions of these siderophores [108]. For example, among *Klebsiella* strains that produce enterobactin and yersiniabactin, 24–40% of the secreted siderophores from urinary isolates consisted of yersiniabactin, whereas only 8% of the siderophores secreted by respiratory isolates consisted of yersiniabactin. Thus it is tempting to speculate that urinary isolates, which secrete more yersiniabactin, may be more successful at niche maintenance at Lcn2-laden mucosal environments, whereas respiratory isolates may be better equipped at exploiting transferrin-rich microenvironments. Therefore, the contribution of the siderophore secretome to *in vivo* bacterial colonization and localization within mucosal microenvironments is an intriguing idea worth exploring.

Bacterial predation

While siderophores restrict local iron availability for siderophilic bacteria, the expression of siderophore receptors also acts as an Achilles' heel by serving as targets for bactericidal agents such as microcins or sideromycins produced by other bacteria [109] [110] [111]. Microcins are antibacterial peptides synthesized by Enterobacteriaceae that target related bacterial species or strains [109]. Some classes of microcins undergo post-translational modifications that include the incorporation of siderophore moieties into the peptide backbone, which enables predation of related bacteria that express the cognate siderophore receptors by microcin producers. Sideromycins are another class of antibacterial agents that can also be delivered through siderophore receptors [110] [111]. Sideromycins are naturally occurring antibiotics conjugated to siderophores and exhibit bacteriocidal activities against siderophilic bacterial taxa such as Enterobacteriaceae and *Staphylococcus aureus*. Both microcins and sideromycins have been efficacious in reducing systemic burdens of Enterobacteriaceae pathogens in mouse models [112] [113]. However, susceptible bacteria also rapidly develop resistance against sideromycins through the acquisition of point mutations within genes encoding the cognate siderophore receptor, resulting in reduced

import of the siderophore-antibiotic conjugate [110]. However, this comes at a cost, where recognition of cognate siderophore-iron chelate is also compromised, thus potentially reducing fitness when iron is a limiting resource. These ecological interactions between siderophilic bacteria and microcin or sideromycin producers present an additional mechanism by which iron can potentially modulate the intestinal microbiota. Thus, the impact of endogenously produced microcins or sideromycins on microbial ecology and consequent host-microbial interactions remains a fascinating avenue for future investigations. Such studies could potentially enable the development of genetically-engineered microcin or sideromycin producing probiotics or synthetic siderophore-antibiotic conjugates as a novel therapeutics to either maintain symbiosis or prevent or treat microbially-driven diseases.

Bacterial virulence and function

In addition to impacting bacterial growth, fluctuations in local iron availability can modulate diverse physiological and functional processes in many bacterial taxa. This can occur through several different global transcription factors that are responsive to changes in intracellular iron concentrations and consequent alterations to the redox state of the cell [114] [43] [44]. Indeed, in addition to modulating cellular iron homeostasis, the iron-responsive transcription factor Fur regulates the expression of genes involved in a diverse array of functions [43] [115] [42] [44], thus providing one of several transcriptional mechanisms by which sensing of the local environment can lead to the modulation of bacterial physiology, virulence and metabolic function. Indeed, increasing iron availability corresponds with significant changes to the metagenome and metabolome of the fecal microbiota in an *in vitro* fermentation model [90]. Similarly, iron impacts the physiology of specific bacteria taxa by affecting the production of extracellular organelles such as flagella and pili, which in turn can modulate bacterial colonization at the epithelial interface and host immune responses [116] [117] [118] [119] [120]. Iron also modulates distinct bacterial interactions with host cells including susceptibility to phagocytosis, epithelial adherence and translocation of bacterial effectors into host cells [121] [122] [123] [119] [120]. Consistent with this, dietary iron interventions in animal models impact the severity of gastrointestinal inflammation and carcinogenesis induced by enteric pathogens such as *Helicobacter pylori*, *Citrobacter rodentium* and *S. enterica* [119] [124]. Taken together, iron can potentially modulate numerous functional aspects of the intestinal microbiota, which in turn may perturb or reinforce the symbiotic relationship between resident bacteria and their host. Moreover, because siderophores and host iron binding protein can also modulate local intestinal iron availability, they may also act as additional novel factors that impact bacterial physiology, virulence and metabolic function.

Siderophores as modulators of host responses

Maintenance of iron homeostasis in the host is complex and is regulated by numerous signaling pathways both at the systemic and cellular levels. Mammalian cells contain intracellular stores of readily exchangeable iron known as the labile iron pool. Perturbations to these intracellular iron stores result in transcriptome-wide changes and altered metabolic functions that modulate a diverse array of cellular responses [36]. Interestingly, in recent

years, siderophores have been demonstrated to perturb local cellular iron homeostasis within host cells. This therefore provides the opportunity for siderophore-producing resident and pathogenic bacteria to modulate local host responses. Interestingly the siderophore desferrioxamine (DFO), synthesized by soil-derived *Streptomyces spp.* and purified for therapeutic use, can also modulate systemic iron homeostasis and has indeed been utilized to treat iron-overload disorders [125]. However, whether siderophores derived from resident enteric bacteria reach systemic circulation and influence systemic iron homeostasis remains an intriguing concept that to our knowledge has not been explored. Thus, this section will solely focus on how siderophores may modulate local cellular iron homeostasis and subsequent host responses. Possible mechanisms by which this may occur are explored in this section and are also summarized in Figure 1.

Host cellular iron homeostasis

As with bacteria, cellular iron status impacts numerous physiological processes within host cells. Interestingly, many studies that have characterized the impact of iron on various host cell functions have utilized the siderophore DFO, to induce a state of iron deficiency. More recently, it was demonstrated that, like DFO, aferric enterobactin alone or in combination with Lcn2 also perturbs the intracellular labile iron pool within respiratory epithelial cells in a manner consistent with iron deficiency [126] [127]. Addition of iron reversed this effect, suggesting that this perturbation was the result of sequestration of intracellular iron sources. Furthermore, it was demonstrated that epithelial cells can internalize the Lcn2-enterobactin complex [126], further supporting the idea that enterobactin can modulate iron homeostasis within host cells. The siderophore yersiniabactin was also shown to disrupt intracellular iron homeostasis when not bound to ferric iron, but salmochelin could not [127], suggesting that not all bacteria-derived siderophores influence host functions in a similar manner. Taken together, these findings introduce the possibility that siderophores produced by resident or pathogenic bacteria in the intestines can modulate host cell iron homeostasis at the mucosa, especially given that enterobactin has been detected in cecal mucus [72].

The intestinal microbiota as a whole can also influence cellular iron homeostasis within enterocytes, epithelial cells that line the intestines. Expression of various proteins involved in iron transport and storage within duodenal and colonic enterocytes differed between germ free and conventionalized mice [60]. This included increased expression of apical iron transporters and decreased levels of the intracellular iron storage protein ferritin within germ free mice, which together are indicative of an iron-starved state. Colonization of germ free mice with *B. theta* alone or in combination with *Fecalibacterium prausnitzii* or with the probiotic strain *Streptococcus thermophilus* resulted in an expression profile of iron transport and storage proteins similar to conventionalized animals [60]. However, it remains unclear whether characteristics unique to these strains modulated enterocyte iron homeostasis or whether general microbial signals such as Toll-like receptor (TLR) ligands are sufficient in restoring an iron replete-like state within enterocytes.

Mucosal immunity

A wide range of host immune responses are intricately linked with both systemic and cellular iron homeostasis. Perturbations to the labile iron pool have been linked with

modulating various forms of innate and adaptive immune functions including proliferation, differentiation and secretion of inflammatory mediators [128]. Processes associated with inflammation, including cytokine production and engagement of TLRs by microbial ligands, also stimulate the production of the hormone hepcidin, which in turn modulates systemic iron homeostasis by limiting dietary iron absorption and increasing intracellular retention of iron [68] [129] [130]. Given that bacteria-derived siderophores can alter the iron status of host cells, this presents the opportunity for siderophilic resident bacteria to modulate host immune responses at the mucosal interface. Furthermore, iron availability is generally decreased with inflammation [52], which favors the expression of siderophore systems by both resident and pathogenic bacteria and thus provides the ideal environment for bacterial modulation of host immune responses.

One host pathway modulated by siderophores is the hypoxia inducible factor (HIF) pathway [127]. Cellular responses to decreased intracellular iron content and low oxygen availability (i.e. hypoxia) converge at the transcriptional regulatory functions of HIF, which can modulate numerous processes including angiogenesis, proliferation, metabolism and cytokine/chemokine-mediated immunity. Under normoxic or iron-replete conditions, the α -subunit of the HIF heterodimer is highly unstable and is targeted for proteosomal degradation by prolyl hydroxylation [131]. In contrast, a decrease in oxygen availability or intracellular iron reduces the activities of iron-dependent and oxygen-responsive prolyl hydroxylase enzymes, thus increasing cellular levels of Hif1 α protein and enabling nuclear accumulation of HIF [132]. Consistent with its ability to perturb the labile iron pool, aferric enterobactin indeed was shown to stabilize HIF1 α within respiratory epithelial cells [127]. Similarly, aferric yersiniabactin and DFO promote the accumulation of HIF1 α within intestinal epithelial cells, an effect that is lost with addition of exogenous iron [133]. Interestingly, intestinal epithelial specific deletion of *Hif1a* resulted in increased mortality to challenge with the yersiniabactin-producing enteric pathogen *Y. enterocolitica* [133], suggesting that HIF1 α -mediated epithelial responses are protective in the context of enteric infection.

A consistently observed response to bacteria-derived siderophores is cytokine secretion from immune and epithelial cells, likely via HIF1 α and other innate immune pathways [134] [135] [136] [126] [137] [127]. Enterobactin and yersiniabactin both induce epithelial secretion of various proinflammatory mediators including the neutrophil-homing cytokine IL-8 [126] [127]. This corresponded with decreased intraluminal infiltration of neutrophils following intranasal infection with an enterobactin-deficient *K. pneumoniae* mutant. Interestingly, this mutant retained the capability of producing yersiniabactin, suggesting that enterobactin uniquely modulates neutrophil homing within the respiratory mucosal environment [137]. Consistent with its inability to perturb cellular iron homeostasis, salmochelin did not induce epithelial cytokine secretion [137] [127]. Numerous studies have also demonstrated that host Lcn2 can potentiate siderophore-mediated induction of epithelial cytokine secretion [126] [137] [127]. Although the underlying mechanism is not well understood, this observation is particularly intriguing considering that Lcn2 secretion is enhanced during intestinal inflammation.

In contrast, siderophores have also been shown to minimize attempts by the innate immune system to establish a noxious and pro-oxidant mucosal environment, which is critical for limiting microbial growth and dissemination. For example, yersiniabactin, and to a lesser extent aerobactin, reduce neutrophil and macrophage production of ROS [138], while DFO treatment decreases *ex vivo* ROS production from colorectal biopsies [139]. Similarly, aferric enterobactin, but not salmochelin or yersiniabactin, uniquely inhibit the bactericidal activities of neutrophil myeloperoxidase (MPO), the protective effects of which are abrogated with the addition of iron [140]. These anti-oxidant effects of enterobactin production were also demonstrated in two mouse models of colitis [140]. In both models, intestinal colonization of an *E. coli* mutant that overproduces enterobactin reduced colonic MPO activity and enhanced its intestinal fitness compared to the parental or enterobactin-deficient strains. These findings demonstrate that enterobactin can shape the *in vivo* mucosal microenvironment to promote conditions favorable for luminal *E. coli* growth. Interestingly, enterobactin overproduction also resulted in reduced overall cecal histopathology [140], likely as a result of decreased MPO activity and consequent host tissue damage. Thus, siderophores appear to have contrasting effects on host immunity by promoting both pro- and anti-inflammatory responses. Therefore, more *in vivo* studies will be required to delineate the precise mechanisms by which distinct siderophores and combinations thereof modulate mucosal immunity, particularly in the context of specific microbial- and inflammation-driven diseases.

Conclusions and Future Perspectives

Microbially-driven pathological processes are now recognized as a key feature of many chronic diseases with complex etiologies, including IBD, cancer, metabolic syndrome and diabetes. High throughput sequencing technologies that have enabled unbiased characterization of the intestinal microbiome have highlighted common compositional and functional changes observed in various pathological states, many of which have been recapitulated through fecal transplantation or gnotobiotic rodent studies. One common feature of dysbiosis in both rodent and clinical studies is a bloom of Enterobacteriaceae, which are well known to secrete and utilize siderophores as an effective mechanism for acquiring iron and have been shown to drive colitic and procarcinogenic responses [102] [32]. Many diseases are also associated with the inappropriate localization of resident bacteria to the mucosal niche. This provides the means for further provocation of proinflammatory responses by the mucosal immune system, thereby perpetuating a state of chronic inflammation. Siderophore-mediated iron transport by resident bacteria such as Enterobacteriaceae may further contribute to this dysbiotic relationship by enhancing microbial growth within the inflamed mucosal environment and exacerbating proinflammatory responses through modulation of local host iron homeostasis. This in turn could serve to further reinforce the establishment of a replicative niche that favors siderophilic bacteria, therefore maintaining a state of dysbiosis. However, siderophore production by beneficial resident bacteria may also contribute to pathogen exclusion and limit pro-oxidant mucosal responses, both of which can minimize intestinal proinflammatory responses. Finally, although not the focus of this review, siderophores have been recently shown to bind other metals such as copper and zinc, which has been implicated in

modulating bacterial fitness and interactions with host cells [40] [141]. Thus it will be interesting to further explore how siderophore production and utilization by specific subsets of resident bacteria within the intestinal microbiota contributes to the development of microbially-driven diseases. The mechanistic findings of such studies will likely provide rationale for the design of novel therapeutics to target siderophore systems, such as synthetic sideromycins, which could in turn disrupt the perpetuating cycle of inflammation and dysbiosis characteristic of many modern, chronic diseases.

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Highlights

- Resident bacteria secrete siderophores to compete for iron in the gut.
- Different siderophores enable bacterial niche formation at unique host sites.
- Bacterial siderophores modulate cellular iron homeostasis and host immunity.
- Siderophores enhance pathogen exclusion and limit pro-oxidant mucosal responses.
- Thus siderophores likely influence microbially-driven pathological processes.

Figure 1. Siderophore-mediated modulation of bacterial communities and host mucosal responses

Depletion of local iron availability induces the upregulation of siderophore systems by resident and pathogenic bacteria and enables efficient scavenging of iron. This impacts the intestinal microbiota through interbacterial competition, local niche formation and bacterial virulence and function. These ecological changes and perturbations of host cellular iron homeostasis impact mucosal immunity, which further modulate the microbial community, host health and disease.

Table 1Common siderophores produced by enteric bacteria^{37, 40, 50}

Bacterial taxa	Siderophores produced	Structure
Enterobacteriaceae (including <i>E. coli</i> , <i>S. enterica</i> , <i>Shigella spp.</i> , <i>Yersinia spp.</i> , <i>Klebsiella spp.</i>)	Enterobactin Salmochelin Aerobactin Yersiniabactin	Catecholate Catecholate (glycosylated enterobactin) Mixed (carboxylate-hydroxamate) Mixed (phenolate)
<i>Pseudomonas spp.</i>	Pyochelin Pyoverdin	Mixed (phenolate) Mixed
<i>Staphylococcus spp.</i>	Staphyloferrin A	Carboxylate
<i>Bacillus spp.</i>	Bacillibactin Petrobactin	Catecholate Mixed (carboxylate-catecholate)
<i>Vibrio spp.</i>	Vibriobactin	Catecholate

Table 2

Intestinal iron concentrations

Reference	Sample type	Intervention	Total iron	Free and/or chelatable iron
Lund 1999 [56]	Human fecal content	Baseline With oral ferrous iron supplementation (100 mg/day)	91–109 mg/kg wet wt 352–369 mg/kg dry wt 278–284 mg/kg wet wt 970–985 mg/kg dry wt	~1.7 mg/kg wet wt (free) ~1.7 mg/kg wet wt (chelatable) ~5.6–7.3 mg/kg wet wt (free) ~6.7–8.4 mg/kg wet wt (chelatable)
Carrier 2001 [57]	Rat fecal content	<u>Inflamed (DSS colitis):</u> 270 mg iron/kg diet 3000 mg pentacarbonyl iron/kg diet 30000 mg pentacarbonyl iron/kg diet	8.7 mg/kg wet wt 61 mg/kg wet wt 668 mg/kg wet wt	2 mg/kg wet wt 16 mg/kg wet wt 93 mg/kg wet wt (combined free and chelatable iron)
Werner 2010 [58]	Mouse ileal tissue and content	<u>Non-inflamed (WT mice):</u> <10 mg Fe/kg diet 180 mg Fe/kg <u>Inflamed (TNF ARE mice):</u> <10 mg Fe/kg 180 mg Fe/kg	~32 mg/kg wet wt ~40 mg/kg wet wt ~32 mg/kg wet wt ~44 mg/kg wet wt	
Deriu 2013 [51]	Mouse fecal content	Non-inflamed Inflamed (<i>Salmonella</i> infection)	950 mg/kg dry wt 300 mg/kg dry wt	
Li 2015 [14]	Mouse colon	<u>Non-inflamed:</u> Luminal content Mucus	0.121 mg/kg 0.027 mg/kg	
Deschemin 2015 [59]	Mouse fecal content	<u>Non-inflamed:</u> Germ free 2 d post conventionalization 40 d post conventionalization	768 mg/kg dry wt 418 mg/kg dry wt 63 mg/kg dry wt	
Lee 2016 [60]	Human fecal content	Baseline With oral ferrous iron supplementation (600 mg/day)	~ 400 mg/kg dry wt ~1700 mg/kg dry wt	