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## Bacterial siderophores in community and host interactions

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### Abstract

Iron is an essential trace element for most organisms. A common way for bacteria to acquire this nutrient is through the secretion of siderophores, which are secondary metabolites that scavenge iron from environmental stocks and deliver it to cells via specific receptors. While there has been tremendous interest in understanding the molecular basis of siderophore synthesis, uptake and regulation, questions on the ecological and evolutionary consequences of siderophore secretion have only recently received increasing attention. In this Review, we outline how eco-evolutionary questions can complement the mechanistic perspective, and help to obtain a more integrated view on siderophores. In particular, we explain how secreted diffusible siderophores can affect other community members, leading to cooperative, exploitative and competitive interactions between individuals. These social interactions in turn can spur co-evolutionary arms races between strains and species, lead to ecological dependencies between them and potentially contribute to the formation of stable communities. In brief, our Review shows that siderophores are much more than just iron carriers: they are important mediators of interactions between members of microbial assemblies and the eukaryotic hosts they inhabit.

### Introduction

Like most other organisms, virtually all bacteria need iron as a co-factor in enzymes to catalyze redox reactions involved in fundamental cellular processes such as respiration, DNA synthesis and protection from reactive oxygen species<sup>1</sup>. Although iron is abundant in the earth crust, its bioavailability is generally low<sup>2,3</sup>. This is because iron mainly exists in its oxidized ferric Fe<sup>3+</sup> state, which is largely insoluble at neutral and basic pH levels, conditions that prevail in many natural habitats. To cope with iron shortage, bacteria have evolved a number of mechanisms to acquire this essential trace element from the environment. These mechanisms include the uptake of iron bound to organic molecules such as citrate or heme, the absorption of iron by membrane-bound uptake systems, and the secretion of siderophores, which are secondary metabolites that scavenge iron from

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environmental stocks by forming soluble Fe<sup>3+</sup>-complexes that are then actively taken up via specific receptors<sup>4–11</sup>.

Almost all known bacterial species produce siderophores, making their secretion the most prevalent mechanism for iron scavenging in the microbial world<sup>4–6</sup>. Siderophores are a chemically diverse group of secondary metabolites<sup>12</sup>. There is an extensive body of work on their chemical structures and the molecular mechanisms of their synthesis, export, uptake, and regulation<sup>1,5–10,12–14</sup>. The enormous interest in the molecular basis of siderophore biology has created an imbalance between what we know on the mechanistic versus the evolutionary and ecological aspects of these vital compounds<sup>15</sup>. While mechanistic knowledge on siderophores grew immensely, evolutionary questions fell behind, probably because of the obvious physiological function of siderophores, having evolved as a means for bacteria to obtain iron for metabolism<sup>1,9,11</sup>. Only relatively recently it was recognized that complementary evolutionary questions, which go beyond physiological aspects, can be addressed, and are important to understand evolutionary and ecological dynamics in bacterial communities and the evolution of bacterial pathogens within hosts<sup>16–24</sup>.

One of these complementary aspects is the question why bacteria have evolved highly diffusible siderophores – a strategy that might successfully solubilize iron, but comes with the problem that siderophores might not find their way back to producers<sup>18,24,25</sup>? Another complementary aspect is highlighted by the observation that siderophores secreted by one cell can have dramatic fitness consequences for other cells in the microbial community, including members of the same and different species<sup>16,17,20,21</sup>. For example, siderophores might not only make iron available for the producer, but also to other individuals with a matching receptor, such that the costs and benefits of siderophore production are distributed across different individuals within the population<sup>16,17</sup>. Alternatively, the binding of iron to siderophores can induce iron starvation in bacteria that lack the matching receptor for uptake<sup>20,26</sup>. Thus, siderophores seem to be important players in mediating interactions between cells of the same and different species.

The aim of this Review is to elucidate these complementary aspects by summarizing the recent insights gained on the ecology and evolution of siderophores and by linking them to the mechanistic aspects of siderophore production. We will start with an overview on the mechanistic aspects of siderophore synthesis, secretion, uptake, and regulation. Although this topic has been extensively reviewed elsewhere<sup>1,6–10,12,13,27</sup>, we will briefly treat it here as it is essential for understanding the ecological and evolutionary consequences of siderophore secretion at the community level. We will then move on to specifically elucidating eco-evolutionary aspects by addressing the following four questions: What are the physical consequences of siderophore secretion for individual cells and bacterial groups? Which types of social interactions are driven by siderophores? How do these social interactions impact evolutionary and ecological dynamics in bacterial communities? How can insights into these eco-evolutionary aspects help us to understand bacteria-host interactions, including the evolution of beneficial functions of the microbiota and detrimental effects by pathogens in infections?

## A mechanistic primer on siderophores

Siderophore (Greek: iron carrier) is a functional term and defines organic ligands that show specificity for iron, enable iron acquisition through specific uptake systems and are regulated in response to iron availability<sup>28</sup> (but also see Box 1 for alternative functions of siderophores). Four chemical types of siderophores can be distinguished based on the moieties involved in iron chelation (Figure 1). These are catecholate, phenolate, hydroxamate and carboxylate types of siderophores, whereby mixtures of the various types are also common<sup>6,12</sup>. Siderophores are typically synthesized by non-ribosomal peptide synthetases (NRPS) or polyketide synthase (PKS) domains that work in concert with NRPS modules. A smaller fraction of siderophores are also produced by pathways that are independent of NRPS and PKS<sup>6,29–33</sup>. Siderophores synthesis via NRPS is the most common form and consists of a multienzyme assembly line, in which amino acids, as well as carboxy and hydroxy acids are built into a peptidic precursor molecule, which is subsequently modified by either NRPS or other enzymes to form the final siderophore. One such modification includes the formation of heterocycles, such as the thiazoline ring arising through the cyclization of cysteine side chains<sup>6,29</sup>. The secretion of siderophores is an energy-driven process and mediated by efflux pumps, which belong to different superfamilies depending on the siderophore type they transport<sup>6</sup>.

The uptake of iron-loaded siderophores differs between Gram-negative and Gram-positive bacteria, mainly because Gram-positive bacteria have no outer membrane across which iron-loaded siderophores must be translocated<sup>1,6,10,13,14,27,34,35</sup>. In Gram-negative bacteria, a common mechanism involves the specific recognition of the iron-loaded siderophore by a  $\beta$ -barrel receptor in the outer membrane. After binding the ligand, the receptor undergoes a conformational change, translocating the iron-loaded siderophore into the periplasm. This process is supported by a TonB-complex, which delivers the energy via the proton motive force<sup>13,34,35</sup>. Transport of the iron-loaded siderophore into the cytosol, where iron reduction occurs, is then typically mediated by an ABC-transporter in the inner membrane<sup>13,34,35</sup>. In some special cases, the iron is reduced in the periplasm and only the ferrous  $\text{Fe}^{2+}$  iron is imported into the cytosol<sup>36</sup>. In Gram-positive bacteria no outer-membrane receptors are required and siderophores are directly imported by ABC-transporters spanning the cell membrane<sup>6,34</sup>. The fate of siderophores after the release of iron is not well understood. In some cases, siderophores can be recycled through specific recycling mechanisms<sup>35,37,38</sup>, whereas other siderophores undergo hydrolysis to release iron<sup>39,40</sup>.

The above described siderophore synthesis, release and uptake mechanisms are under tight regulatory control aimed at maintaining iron homeostasis within the cell. The most common key regulator is Fur (ferric uptake regulator), a transcriptional repressor of siderophore synthesis and regulatory activator genes<sup>5,41,42</sup>. When intracellular iron stores are replenished, Fur binds ferrous iron in the cytosol and this complex then binds to the promoter region of either siderophore synthesis<sup>42</sup> or regulatory activator genes<sup>43</sup> to block their transcription. Under iron limitation, repression by Fur is released and siderophore synthesis induced. Another level of siderophore regulation occurs when iron-loaded siderophores enter the cell. Here, extracytoplasmic function sigma factors or two-component systems serve as checkpoints, triggering increased siderophore synthesis. This positive

feedback loop is interpreted as a signal to cells that iron scavenging by siderophores is a successful course of action<sup>6,44,45</sup>.

## Consequences of siderophore secretion

### Siderophore secretion from the perspective of an individual cell

The physical consequence of siderophore secretion is that the molecules can diffuse away, beyond the reach of producers, and might thus not deliver benefits back to producer cells. One key evolutionary question related to the physical consequence of loss through diffusion is why bacteria have evolved diffusible siderophores in the first place and do not solely rely on membrane-bound iron uptake systems, which occur in many taxa<sup>46,47</sup>. Mathematical models have tackled this question on multiple fronts<sup>24,25,48–51</sup>. One insight gained from these theoretical analyses is that membrane-embedded systems seem superior over siderophores in planktonic cells living in unstructured environments. This advantage arises because a lack of spatial structure results in high siderophore losses through diffusion, and at the same time leads to a more homogeneous iron distribution, which makes iron easily accessible by membrane-embedded systems (Figure 2a)<sup>24,25</sup>. When bacteria are attached to surfaces, siderophore secretion enables cells to chelate iron beyond the local pool, which in turn might become rapidly depleted if iron is taken up by membrane-embedded systems (Figure 2b). Yet for single cells without neighbours, most of the additional iron chelated by siderophores is still likely lost due to diffusion and membrane-embedded system might still perform reasonably well<sup>24</sup>. The key advantage of siderophores seems to kick in when iron resources are clumped (for example, in mineral particles) and bacteria might not necessarily be located near the iron source (Figures 2c+d)<sup>24,25,48</sup>. In this scenario, siderophores enable increased solubilisation of iron, fostering a more homogenous distribution of this nutrient and making it accessible to both planktonic and sessile cells. Conceivably, membrane-embedded systems perform poorly when iron is clumped, especially when cells are sessile and physically disconnected from the iron source (Figure 2d).

Despite these considerations, siderophore loss due to diffusion can still be substantial and compromise bacterial fitness. One important factor that has been shown to limit loss through diffusion and increase benefits of siderophores is environmental structure, a factor that takes effect when bacteria either live in physically structured habitats (for example, on organic or inorganic particles) or in viscous medium (for example, in the mucus of individuals with cystic fibrosis)<sup>49–52</sup>. Indeed, a study investigating bacterioplankton<sup>17</sup> showed that siderophore-mediated iron scavenging is beneficial in *Vibrio* spp. attached to organic particles, which represent a structured island in the otherwise highly diffusive marine environment. Another factor that contributes to the control of loss through diffusion is the chemical structure of the siderophore molecule itself<sup>18,53,54</sup>. A comparative analysis revealed that many marine bacteria produce large amphiphilic siderophores that can, at least partially, be embedded into the cell membrane<sup>18</sup>, an adaptation that clearly helps to retain siderophores and reduce loss through diffusion in a highly diffusive environment like the open ocean<sup>53,54</sup>. Although such amphiphilic siderophores can also occur in species living in relatively structured environments such as soil<sup>55</sup>, they are much rarer in these environments,

presumably because the physical structure of the habitat itself limits the diffusion of molecules<sup>18</sup>.

### Siderophore secretion from the perspective of a group

An important feature of bacterial life is that cells are hardly ever alone, but rather grow in groups, often in biofilms attached to surfaces in soil, hosts, and particles in aquatic environments<sup>56–58</sup>. This has important consequences for the use of siderophores (and other secreted goods), as cells do not necessarily rely on their own siderophores for iron scavenging, but can also benefit from the molecules secreted by their clonemates (Figure 3)<sup>59</sup>. Siderophore sharing is thus a powerful mechanism to limit the negative consequences of siderophore loss through diffusion, and could be an additional factor that determines whether diffusible siderophores can gain an edge over membrane-embedded iron uptake systems. This effect could be important even in habitats in which iron is relatively homogeneously distributed and cells are motile, as long as local cell density is sufficiently high (Figure 3)<sup>25,49,60</sup>.

## Social interactions and siderophores

### Cooperation

Because secreted molecules can be shared between cells siderophore production is often considered a form of cooperation<sup>61</sup>. Cooperative behaviours involve an actor generating benefits for another individual, with such interactions having at least partly been selected for this purpose<sup>15,62</sup>. According to evolutionary theory two forms of cooperation can be distinguished depending on whether the direct (lifetime) fitness consequence for the actor is negative (altruistic cooperation) or positive (mutually beneficial cooperation)<sup>62</sup>. For both types of cooperation, the inclusive fitness (that is, direct fitness benefits plus indirect benefits of assisting relatives) must be positive in order to be selected for<sup>15</sup>. These evolutionary aspects have been very well studied for the siderophore pyoverdine produced by *Pseudomonas aeruginosa*<sup>16</sup>, for which both the cost of production<sup>63,64</sup> and the benefit to others<sup>65</sup> have been demonstrated. The consensus across numerous papers<sup>64–67</sup> studying the cooperative nature of this system is that siderophores are public goods and their production typically represents a mutualistic form of cooperation, providing direct benefits to both the producers and the recipients (Fig. 4a). Similar evidence emerged from studies on other siderophores including pyochelin from *P. aeruginosa* and *Burkholderia cenocepacia*<sup>68,69</sup>, ornibactin from *B. cenocepacia*<sup>69</sup> and enterochelin from *Escherichia coli*<sup>70</sup>.

The evolutionary origin of siderophore secretion is unknown, and a key question is whether the relative importance of self-directed benefits versus indirect benefits to others has changed over evolutionary time. One plausible scenario is that secretion was initially selected for the direct fitness advantages it provides over membrane-bound iron-scavenging systems under the conditions discussed in Fig. 2. Then perhaps later on, regulatory mechanisms evolved that enabled fine-tuned regulation of siderophore production to increase fitness benefits to others. Such adaptations could include siderophore-mediated signalling<sup>44</sup> to adjust production levels to the local neighbourhood, siderophore recycling<sup>37,71</sup>, the specific upregulation of siderophore production to compensate for the

presence of non-producers<sup>65,66</sup>, and the evolution of more diffusible molecules to increasingly benefit others<sup>18</sup>.

## Cheating

Although cooperation can generate substantial fitness benefits for all individuals involved, it might not be evolutionarily stable. The reason is simple: every cooperative act can be exploited by cheaters that save the costs of cooperation but freeride on the cooperative benefits generated by others<sup>72,73</sup> (Figure 4b). The tug-of-war between cooperators and cheaters can result in the so-called ‘tragedy of the commons’<sup>72,74–76</sup>, in which cooperation, although maximising group benefits, collapses due to exploitation by selfish cheaters. These evolutionary concepts were the starting point for a wealth of studies examining interactions between cooperative siderophore producers and non-producers acting as cheaters<sup>16,77–81</sup>. Again, work on the siderophore pyoverdine from *P. aeruginosa* took a lead role. This body of work revealed that the collapse of cooperation can indeed occur in certain setups<sup>16,82–84</sup>, but under many conditions producers are also able to co-exist with non-producers<sup>76</sup>. This finding spurred interest in understanding the mechanisms that contribute to the maintenance of cooperative siderophore production. One mechanism identified is increased spatial structure, which limits both cell dispersal and siderophore diffusion, and thereby ensures that siderophores are more likely shared between clonemates than cheaters<sup>52,67,79,85</sup>. In addition, fine-tuned regulatory circuits and recycling mechanisms, which optimise the benefit-to-cost ratio of siderophore investment, also play a role<sup>37,71,86,87</sup>. Furthermore, genetic architecture constrains cheater success, as found in the case of *P. aeruginosa* in which pyoverdine non-producers up-regulate the production of the secondary siderophore pyochelin<sup>68</sup>, and in *B. cenocepacia* in which ornibactin mutants can no longer produce the receptor required for ornibactin uptake<sup>69</sup>. Low cell density can also limit the cheaters’ access to siderophores<sup>60,70</sup>. Finally, negative-frequency dependent selection leads to cheaters only experiencing relative fitness benefits when rare but not when common, as the exploitable pool of siderophores depletes when cooperator frequency declines<sup>88,89</sup>.

While this work has fundamentally improved our understanding of social interactions between producers and non-producers under laboratory conditions, there is increasing evidence that the observed social patterns also matter in natural systems. Specifically, siderophore non-producers were found in soil<sup>20,90</sup>, freshwater<sup>20</sup>, marine<sup>17</sup>, hospital<sup>91</sup> and host-associated<sup>19,92</sup> habitats. Moreover, it was demonstrated that in certain cases non-producers can act as cheaters and exploit foreign siderophores<sup>17,20,90,93</sup>. Although non-producers can evolve for other reasons than cheating (for example, in habitats with high iron availability)<sup>94,95</sup>, the cumulative evidence suggests that siderophore-mediated cooperation and cheating are important features of bacterial communities.

## Competition

The discussed scenarios of cooperation and cheating typically involve interactions among closely related individuals that share the same siderophore system. However, bacteria often live in diverse consortia with interacting strains that likely have different siderophores, each requiring a specific cognate receptor for iron uptake<sup>12,18</sup>. For these scenarios, evolutionary theory predicts that siderophores can function as competitive agents against other

species<sup>26,96</sup>. The reasoning is straightforward: in an iron-limited environment, siderophores can lock iron away from competitors that do not have the matching receptor for uptake and thereby reserve iron for the cognate producers (Figure 4c). The consequences of locking iron away were found to be particularly dramatic for non-producers, which have no opportunities to enter the competition with their own siderophores and thus experience severe iron starvation<sup>20</sup>(Figure 4c). If two competing strains both produce their own specific siderophore (Figure 4d) the outcome of the competition can depend on a number of factors, including the amount of siderophores produced, the iron-binding properties of the competing siderophores and the plasticity and temporal dynamics of siderophore production. For example, experiments with *Pseudomonas* and *Burkholderia* species showed that the relative differences in iron-binding properties between siderophores indeed determined the outcome of the competition<sup>21,97</sup>. At the regulatory level, it was found that *P. aeruginosa* up-regulates siderophore production in competition with *Staphylococcus aureus*<sup>98</sup>, whereas, in competition with *B. cenocepacia*, *P. aeruginosa* increased not the overall production of pyoverdine but the rate of production during the early growth phase, which conferred *P. aeruginosa* a competitive advantage<sup>99</sup>.

## Evolutionary and ecological dynamics

### Co-evolutionary arms races

Because social interactions driven by siderophores can have substantial fitness effects, one would predict (based on evolutionary theory) that these interactions should trigger reciprocal evolutionary adaptations in interacting strains or species<sup>100</sup>. In other words, we would expect evolutionary transitions between the scenarios outlined in Fig. 4. Aspects of these transitions have been well studied in *Pseudomonas* bacteria and their siderophore pyoverdine. A first and frequently observed transition can occur in populations of siderophore producers, which are invaded by *de novo* arising non-producer mutants. This invasion induces a shift from cooperation to cheating at the population level (Figure 4a to 4b)<sup>68,78,98</sup>. Another transition could be induced by siderophore producers that adapt to the presence of cheaters. In *P. aeruginosa* such adaptations were found to include reduced pyoverdine production<sup>82,101</sup>, increased investment in alternative iron acquisition mechanisms<sup>102</sup>, and possibly the evolution of new variants of siderophore-receptor pairs that restrict access of the cheaters to siderophores<sup>103</sup>. The evolution of siderophore-receptor diversity has attracted a lot of attention, especially in *Pseudomonas* spp., in which dozens of different pyoverdine-receptor variants exist<sup>20,103–107</sup>. Although a direct proof that cheating drives pyoverdine-receptor diversification is still lacking, diversification could induce the transition from cheating to locking iron away from non-producers (Figure 4b to 4c). Non-producers could finally close the co-evolutionary cycle by evolving receptors that match the modified siderophores, or by acquiring a matching receptor via horizontal gene transfer. Indeed, natural *Pseudomonas* spp. isolates from soil and freshwater habitats (non-producers and producers) had a whole array of different pyoverdine receptors, suggesting that bacteria have evolved strategies to exploit multiple pyoverdine types produced by different community members<sup>20,108,109</sup> with regulatory mechanisms being in place to selectively up-regulate the corresponding receptors<sup>106</sup>.

Antagonistic co-evolution should also occur between two species or strains with different siderophore systems (Figure 4d), in which reciprocal siderophore competition could select for increased siderophore production and/or novel siderophore variants with increased iron affinity<sup>26</sup> or altered diffusion properties<sup>11,18</sup>. Moreover, the acquisition of heterologous siderophore receptors through horizontal gene transfer seems to be a common strategy to increase competitive abilities. For example, *P. aeruginosa* has receptors for the bacterial siderophores enterobactin and ferrioxamine, and a receptor for the fungal siderophore ferrichrome<sup>45,110</sup>. To add another level of complexity, simultaneous co-evolutionary arms races between different siderophore producers and non-producers in diverse natural communities might offer a potential explanation for why bacteria often produce multiple types of siderophores (for example, *Vibrio anguillarum* produces anguibactin and vanchrobactin<sup>111</sup>; *P. aeruginosa* produces pyoverdine and pyochelin<sup>112</sup>; *B. cenocepacia* produces ornibactin and pyochelin<sup>113</sup>). Specifically, the production of different siderophores might increase competitiveness, because it should decrease the likelihood that a competitor can exploit the full siderophore repertoire of a producer. Taken together, these considerations demonstrate that siderophore-mediated iron acquisition strategies are not fixed but in flux, likely driven by the bacterial battle for iron.

### Ecological dependencies

Although cheating provides a short-term benefit to siderophore non-producers, it can also make them dependent on producers in the long run, especially in environments in which siderophores are essential for growth<sup>88,89,114</sup>. Extreme cases of such ecological dependencies have been described for marine bacteria<sup>115</sup>: several unculturable species became culturable upon the supplementation of exogenous siderophores. Siderophore-based dependencies typically result in negative frequency-dependent selection (Figure 5a), as the relative fitness of non-producers declines when they become more common in the population, leading to a stable co-existence between donating siderophore producers and dependent non-producers<sup>88,89</sup>.

### Non-transitive community dynamics

For simplicity, we have introduced siderophore-mediated social interactions as pairwise interactions between two strains and/or species (Figure 4). This obviously does not capture the diversity of bacterial strategies existing in complex communities that harbour many different types of siderophore non-producers and producers with different siderophore-receptor pairs. From an ecological perspective, the co-occurrence of a diverse set of social strategies could give rise to non-transitive population dynamics, in which the co-existence of strains and species can be fostered without any evolutionary change<sup>116–118</sup> (Figure 5b). Laboratory studies with experimental communities with either different *P. aeruginosa* strains<sup>97</sup> or *P. aeruginosa* and *B. cenocepacia* assemblies<sup>21</sup> indeed described such non-transitive population dynamics. In both cases, interacting strains combined the social interactions described in Figs. 4b-d: a strain with a relatively weak siderophore (i) locked iron away from a non-producer, but (ii) was dominated by a strain producing a strong siderophore, which in turn (iii) was exploited by the non-producer. Because each of the strains outcompeted one community member but lost against the other one, there was no overall winner, such that all strains could co-exist. Although these studies provide proofs of



concept, the role these non-transitive interactions might have in shaping the composition and stability of natural species assemblies still remains to be explored.

### Specialization and cross-use

Although most of the studies referred to in this Review focussed on siderophores alone, we should not forget that bacteria often secrete other compounds, including enzymes (for example, proteases<sup>119</sup>), biosurfactants<sup>120</sup> and other metallophores<sup>28</sup>, which can also potentially be shared as public goods among cells<sup>59,121</sup>. Thus, arguments have been raised that siderophore non-producers might only be cheaters with regard to this public good, whereas they are cooperators for other public goods<sup>81,122</sup>, and that they might exchange their public goods with siderophore producers in a mutually beneficial way<sup>94,123</sup>(Figure 5c). Such division of labour could eventually lead to mutual dependencies<sup>124</sup>. Although laboratory studies on genetically engineered or experimentally selected communities demonstrated the benefits of mutualistic cross-feeding interactions involving the exchange of nutrients such as amino acids<sup>125–127</sup>, evidence for mutualist interactions involving siderophores is scarce. There is, however, one convincing example involving marine bacteria that traded siderophores for fixed carbon with an algal phytoplankton<sup>128</sup>. The scarcity of mutualistic interactions involving public goods (apart from amino acids) might match theoretical work suggesting that the conditions favouring mutualism between different bacterial strains and/or species are restrictive<sup>129</sup>. In further support, empirical observations show that competitive interactions predominate over cooperative interactions in bacterial communities<sup>130</sup>.

### Host interactions

As many bacteria associate with multicellular organisms including plants, animals and humans<sup>131</sup>, the above described siderophore-mediated social interactions and population dynamics can potentially have consequences for the host, too. The role bacterial siderophores can play for hosts was first recognized in the plant rhizosphere<sup>132,133</sup>. This habitat harbours diverse microbial communities<sup>134</sup>, and it was found that siderophores of commensal or mutualistic species can reduce the growth of plant pathogens<sup>132,133,135,136</sup>. Although the exact mechanism of pathogen suppression still remains to be elucidated, there is evidence that siderophore-mediated competition is involved as described in Figures 4c+d, which leads to plant-protecting bacteria locking iron away from pathogens<sup>133</sup> (Figure 6a). Similar mechanisms of pathogen suppression might operate in gut microbiota. For example, a probiotic *E. coli* strain, which occurs in the human gut, reduced the intestinal colonization by *Salmonella enterica* subs. *enterica* serovar Typhimurium through siderophore-mediated iron competition<sup>137</sup> and through the targeted antibacterial activity of the siderophore mimic microcin<sup>138</sup>.

Another set of studies has examined whether siderophore-mediated evolutionary dynamics occur within hosts, especially in the context of infections. Iron is limited within the host as it is bound to proteins such as transferrin and lactoferrin<sup>6,9</sup>. Thus, pathogens typically have to deploy siderophores to scavenge iron from host tissues, rendering siderophores important determinants of virulence<sup>6,9,139</sup>. Because of their importance for bacterial growth, it has

been suggested that cheating could occur during infections, when de novo evolved non-producers exploit the siderophores of producers, inducing cooperator-cheater dynamics that could steer infections towards lower virulence<sup>23,61,140</sup>. This scenario has been studied in detail in chronic lung infections with *P. aeruginosa* in individuals with cystic fibrosis<sup>19,22,92,141</sup> (Figure 6b). Over time, the following sequence of evolutionary events were repeatedly observed across patients: infections were established by wildtype producers of the siderophore pyoverdine; mutations in the pyoverdine regulon gave rise to pyoverdine non-producers; pyoverdine non-producers fixed in a high proportion of the patients; mutations accumulated in the pyoverdine receptor genes of non-producers to alleviate the costs of receptor production; and finally the upregulation of the Phu system occurred, a private iron acquisition system extracting iron from host heme molecules. One way to interpret this sequence of events is that non-producers evolved as cheaters, taking over the cooperative population, but because iron is still limited in the lung, siderophore non-producers eventually switched to an alternative membrane-bound iron acquisition system to compensate for the inability to make siderophores. However, it is also important to consider that the lung environment itself changes over time<sup>142–144</sup>, and the increasing level of anaerobiosis has been proposed as an additional factor inducing a shift in the use of siderophore-bound Fe<sup>3+</sup> to heme-bound Fe<sup>2+</sup><sup>145</sup>.

Finally, potential cheating in infections is not limited to within-species interactions, as evidenced by a study on inter-species competition in the gut of the nematode *Caenorhabditis elegans*<sup>146</sup>. In this study, the commensal *Enterococcus faecalis* exploited siderophores produced by the pathogen *Staphylococcus aureus*. In response to exploitation, *S. aureus* down-regulated siderophore production, which in turn reduced virulence as a by-product.

## Conclusions

Nobel Prize laureate Nikolaas Tinbergen explained in one of his seminal papers that every biological phenomenon can be approached by four different questions, which belong to two main categories: proximate and ultimate questions<sup>147</sup>. Proximate (how) questions address mechanistic and developmental aspects of the biological phenomenon, whereas ultimate (why) questions address evolutionary (phylogenetic) and adaptive questions. Bacterial siderophores are a perfect example of how this concept of complementary questions can be used to obtain an integrative view on this important class of secreted metabolites. While there was initially a focus on the proximate aspects of siderophores, exploring the mechanistic aspects of synthesis, secretion, uptake and regulation, ultimate questions covering evolutionary and adaptive aspects have caught up over the last decade. The aim of our Review was to elucidate these ultimate aspects and link them to the mechanistic aspects of siderophore production. The emerging view of this integrative approach is that siderophores are more than just iron carriers: they are important mediators of interactions between members of microbial assemblies and their eukaryotic hosts.

Despite these novel insights there are still fundamental gaps in our knowledge. One obvious shortcoming is that the current research on eco-evolutionary aspects of siderophore production is biased towards *Pseudomonas* species, and one particular siderophore — pyoverdine. We clearly need additional study systems to verify whether the insights gained

from pseudomonads are representative of general siderophore biology, transcending bacterial phylogeny, and habitat and host types. Valuable contributions in this context are the evolutionary studies on aerobactin and vibrioferrin in *Vibrio* sp.<sup>17</sup>, enterochelin in *E. coli*<sup>70</sup>, ornibactin and pyochelin in *B. cenocepacia*<sup>69</sup>, and a comparative study across 189 different siderophores<sup>18</sup>, which could confirm general eco-evolutionary roles of siderophore but also helped to add new elements to the picture. Along a similar line, there is a bias towards studies on simplified laboratory systems. Although these systems offer many advantages as they are tractable and easy to manipulate, we must also embrace more natural systems, studying interactions between more than two strains and species at a multi-dimensional scale and including other social traits in addition to siderophores. Only such integrative approaches will enable us to understand assembly patterns and the functioning (for example, the stability or productivity) of complex microbial consortia. Finally, we might also want to go back in time and think about the evolutionary origins of siderophores. The two-pronged and co-ordinated evolution of a secreted iron-chelating ligand and a receptor for the purpose of iron uptake is an extremely unlikely scenario. A more plausible (yet speculative) possibility is that siderophores have evolved prior to the Great Oxygen Event<sup>148</sup> as agents to prevent iron intoxication during an era when iron was highly soluble. Only after the Great Oxygen Event when iron availability became drastically reduced, bacteria might have evolved receptors to retrieve this newly limiting resource. In the light of all these considerations, we believe that there is still much to be discovered and we have so far only explored the tip of the iceberg of siderophore biology.

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## Glossary

### Siderophores

secondary metabolites with high affinity and specificity for iron that function as an organic ligands, serve the purpose of iron acquisition, and are regulated in response to the producer's need for iron.

### Biofilms

aggregates of microorganisms that are embedded within a self-produced matrix of extracellular polymeric substances and that adhere to each other and/or a surface.

### Cooperation

a social behaviour which provides a benefit to another individual, and which has evolved and/or is currently maintained (at least partly) because of its beneficial effect on the recipient.

### *Pseudomonas aeruginosa*

a metabolic versatile, ubiquitous, rod-shaped, Gram-negative bacterium that can opportunistically infect plants, animals and humans, and is known for its high intrinsic resistance to antibiotics.

**Public goods**

costly resources that not only benefits the producer, but also other members of the population or local community.

**Tragedy of the commons**

a situation in which cooperation would be beneficial in the long term, but cooperation breaks down because individuals selfishly pursue their own short-term interests.

**Negative frequency dependent selection**

an evolutionary process by which the relative fitness of a phenotype is high when it occurs at low frequency in the population, but decreases as it becomes more common relative to other phenotypes.

**Cheating**

exploitation of a cooperative behaviour by an individual that does not cooperate (or cooperates less than its fair share), whereby the cheating individual reaps the benefits of cooperation at the expense of the cooperating individual.

**Competition**

a situation that arises when two or more individuals of the same or different species strive for the same limited resource, resulting in immediate costs for all involved individuals.

**Horizontal gene transfer**

the transfer of genetic material from one individual to another individual (of the same or different species) that does not involve the vertical transmission of DNA typical for cell division and reproduction.

**Co-evolutionary arms races**

evolutionary tug-of-war between competing strains or species, whereby adaptations in one party select for counter-adaptations in the other party.

**Non-transitive population dynamics**

population dynamics arising from non-hierarchical circular competitive relationships between species, in which each species is both superior and inferior towards different community members, with no overall winner existing in the population.

***Metallophores***

a secondary metabolite with high affinity and specificity for a given metal. It functions as an organic ligand, serves the purpose of acquiring the metal in question, and is regulated in response to metal limitation.

**Division of labour**

the division of a task that occurs when cooperating individuals specialize to carry out specific subtasks.

### Cross-feeding

a nutritional interdependence between different strains or species, in which each species feeds on the metabolic products released by the other species.

### Virulence

the damage caused to the host by a parasite or pathogen, measured as the decrease in host fitness.

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**Box 1****Alternative functions of siderophores**

Siderophores might have alternative functions in addition to iron scavenging. From an evolutionary point of view, it is not always clear whether these additional functions reflect mere by-products of iron chelation or adaptations in their own right.

**Non-iron metal transport**

Siderophores can bind metals other than iron, including zinc, manganese, copper and nickel. In some cases, these metal-siderophore complexes do not enter the cell<sup>149,150</sup>, possibly indicating that their formation is a by-product of iron acquisition. However, there are also cases of siderophores that function as multi-purpose metallophores: for example, *Yersinia pestis* produces yersiniabactin and this siderophore scavenges zinc, copper and nickel, in addition to iron, for bacterial metabolism<sup>151,152,153,154</sup>.

**Toxic metal sequestration**

Heavy metals can be lethal at high concentrations<sup>155</sup>. Because siderophores typically bind heavy metals without shuttling them into the cell, they can effectively detoxify the local environment<sup>150,156</sup>. This bioremediation mechanism can be adaptive and specifically upregulated in response to pollution<sup>150,157</sup>. Moreover, it constitutes a cooperative behaviour as producers detoxify the environment for everybody<sup>158</sup>.

**Signalling**

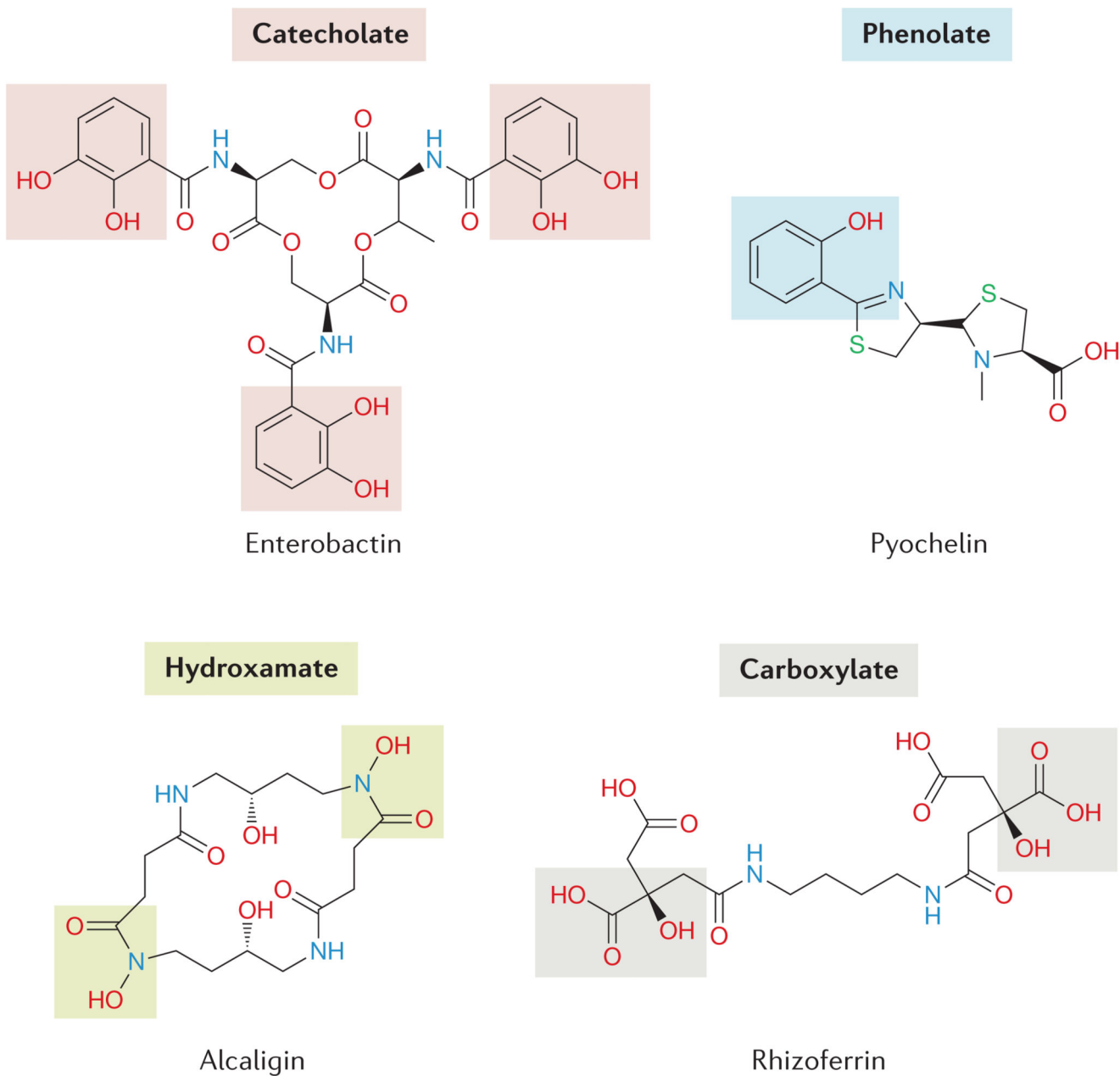
Siderophores can function as signals and thereby regulate their own production, and induce the expression of virulence factors and other components of the iron acquisition pathway, whose regulation is linked<sup>44</sup>. Additionally, siderophores can be cues in inter-species interactions. For example, siderophores produced by one species can stimulate sporulation or induce the production of endogenous siderophores in another species<sup>159,160</sup>.

**Protection from oxidative stress**

Siderophores can offer protection against reactive oxygen species (ROS) created by UV-radiation or antibiotic stressors<sup>161–164</sup>. For example, pyoverdine can absorb UV-radiation extracellularly and thus reduce ROS-generation<sup>161</sup>, but it can also chelate ferrous iron that is released from ROS-damaged Fe-S clusters, thereby preventing the iron-catalyzed reaction of H<sub>2</sub>O<sub>2</sub> to harmful hydroxyl radicals within cells<sup>164</sup>.

**Antibiotic activity**

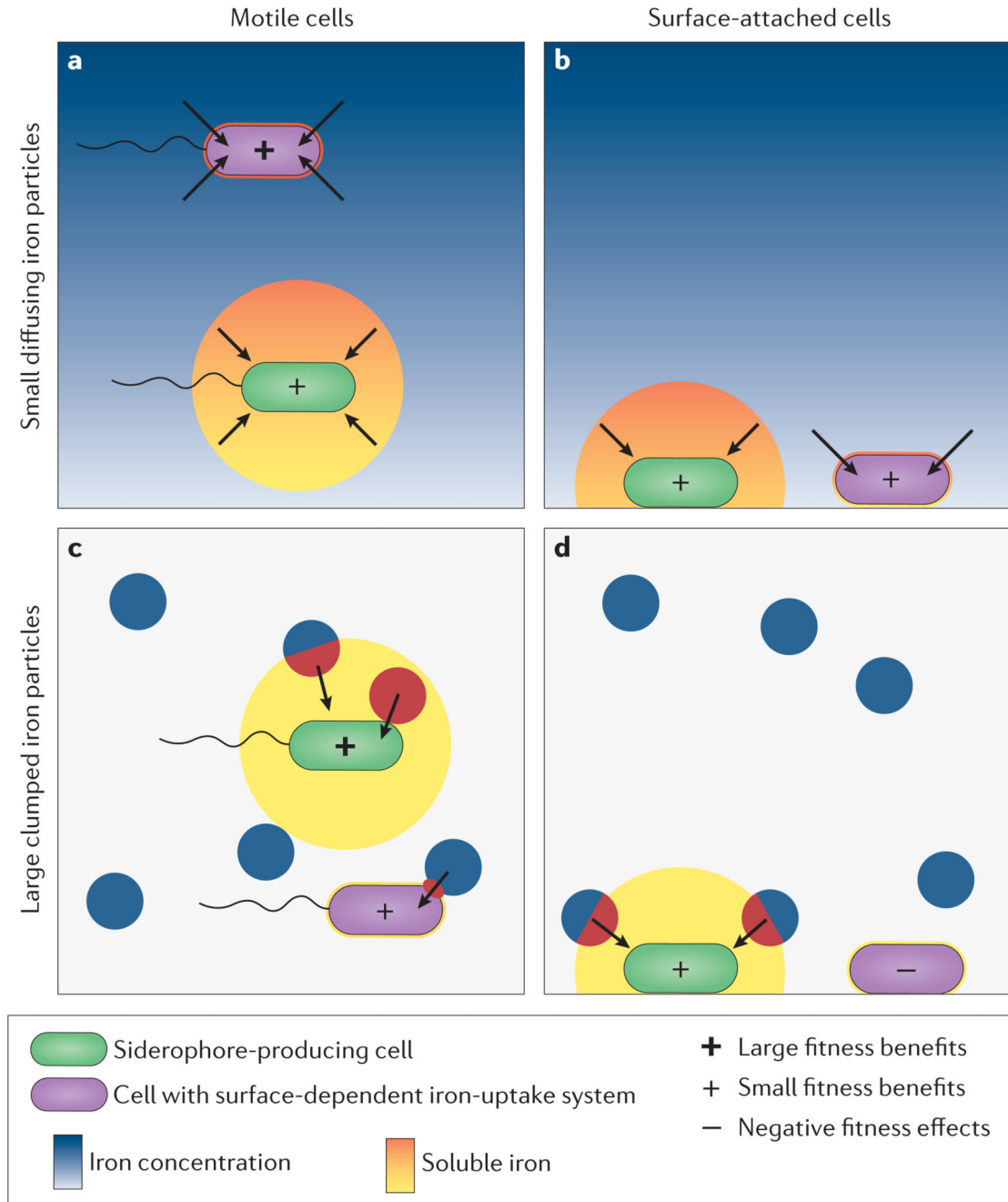
Siderophore receptors are gateways into the cytoplasm, and some bacteria exploit this weak spot by deploying sideromycins, a class of antibiotics in which a bactericidal ‘warhead’ is attached to a siderophore<sup>138,165</sup>. The specificity of sideromycins is largely determined by the prevalence of the respective cognate siderophore receptor, and their activity can be antagonized by siderophores that compete for the same receptor.



**Figure 1. Representative examples of the four main chemical classes of siderophores.**

Siderophore is a functional term and includes a chemically diverse group of molecules. The four main types are distinguished based on the moieties involved in iron chelation, which entail catecholate, phenolate, hydroxamate and carboxylate functional groups (grey shadings). Siderophores with mixtures of functional groups are also common.

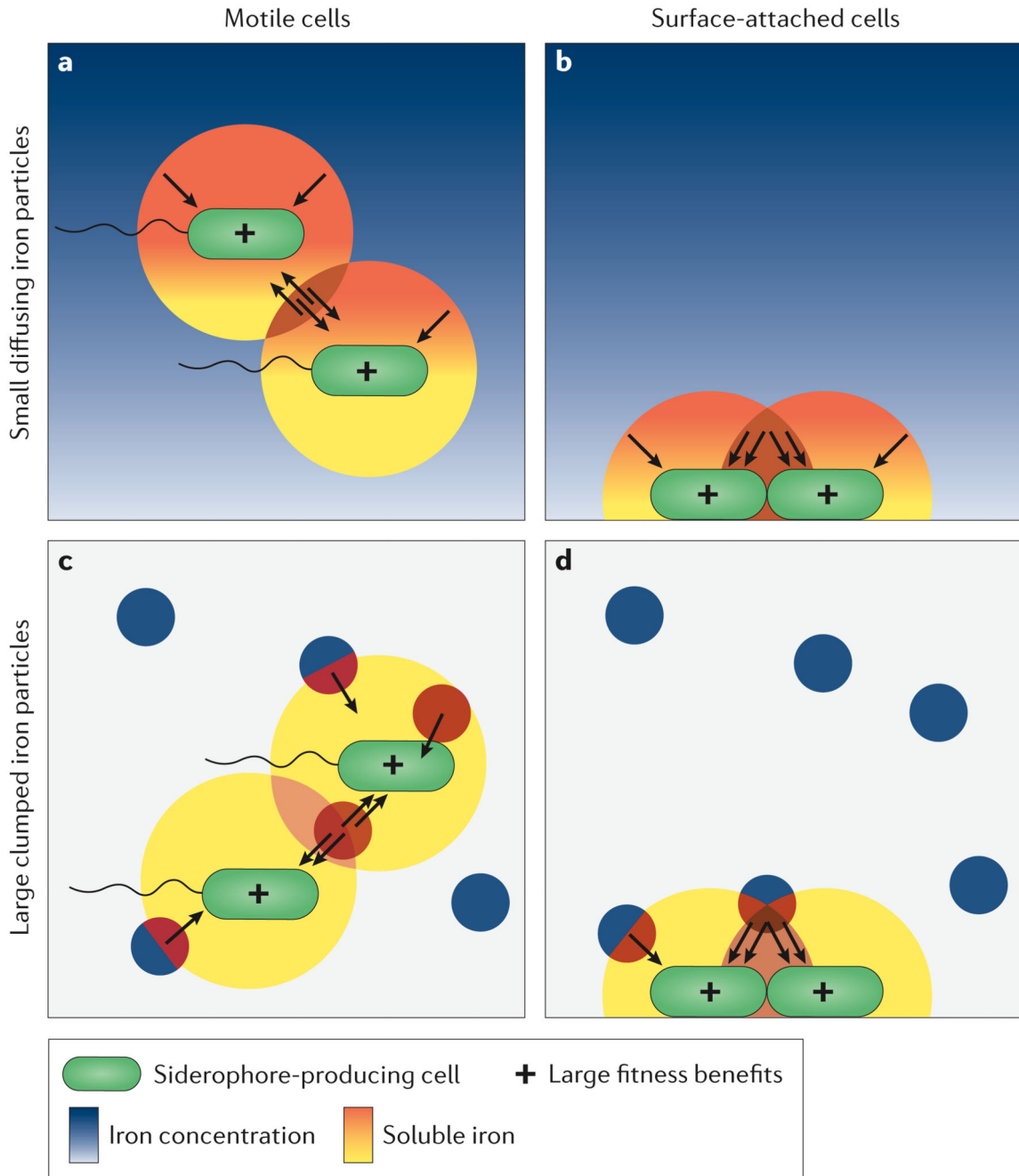
Representative siderophore examples include enterobactin (which is produced by *Escherichia coli*, for example), pyochelin (which is produced by *Pseudomonas aeruginosa*, for example) featuring a heterocycle (thiazoline) ring, alcaligin (which is produced by *Bordetella pertussis*, for example) and rhizoferrin (which is produced by *Ralstonia pickettii*, for example). Note that the same siderophore can be produced by different species<sup>18</sup>.



**Figure 2. Comparing siderophore-dependent and surface-dependent iron-uptake systems.** Considerations of why bacteria have evolved siderophore-mediated iron scavenging systems (green cells), where siderophores can be lost due to diffusion, as opposed to surface-dependent iron uptake mechanisms (purple cells), where benefits are secured. We consider cases in which iron is either relatively homogeneously distributed (as small particles along a blue-shaded gradient) or occurs clumped in large particles (blue circles). Siderophores increase the range across which iron can potentially be sequestered (yellow area) and the amount of iron that is brought into solution (orange-red gradient), but are at the risk of not

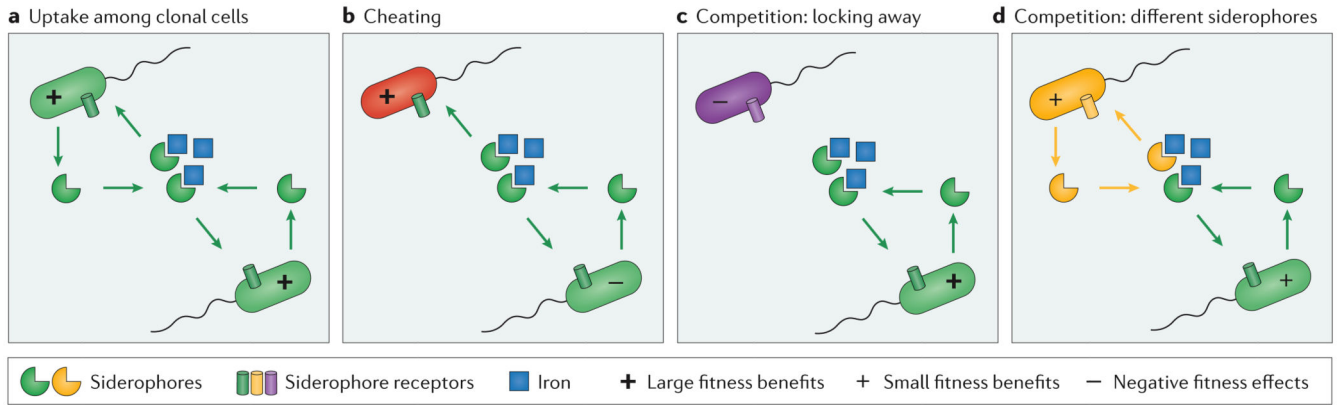
finding their way back to producers (arrows). The siderophore's reach of action (yellow area) depends on the viscosity of the environment and the chemical diffusion property of the siderophore. Emojis inside cells depict the relative fitness consequences of the two iron-scavenging strategies. **a** | When iron is homogenously distributed and cells are motile, surface-dependent iron uptake mechanisms are efficient for iron scavenging, and siderophores unlikely confer an advantage. **b** | When iron is homogenously distributed, but cells are attached to surfaces, siderophores can access iron beyond the local pool, potentially making it available for bacterial metabolism. However, many siderophore molecules might get lost due to diffusion. **c** | When iron is clumped and cells are motile, bacteria with surface-dependent iron uptake mechanisms must necessarily make contact with the iron source to be able to take up this nutrient, whereas no direct cellular contact is required with siderophores and more distant iron pools can be accessed. **d** | When iron is clumped and cells are non-motile, siderophores might be the only way to obtain iron. These considerations suggest that siderophore production is most beneficial when iron resources are clumped and cells are non-motile.





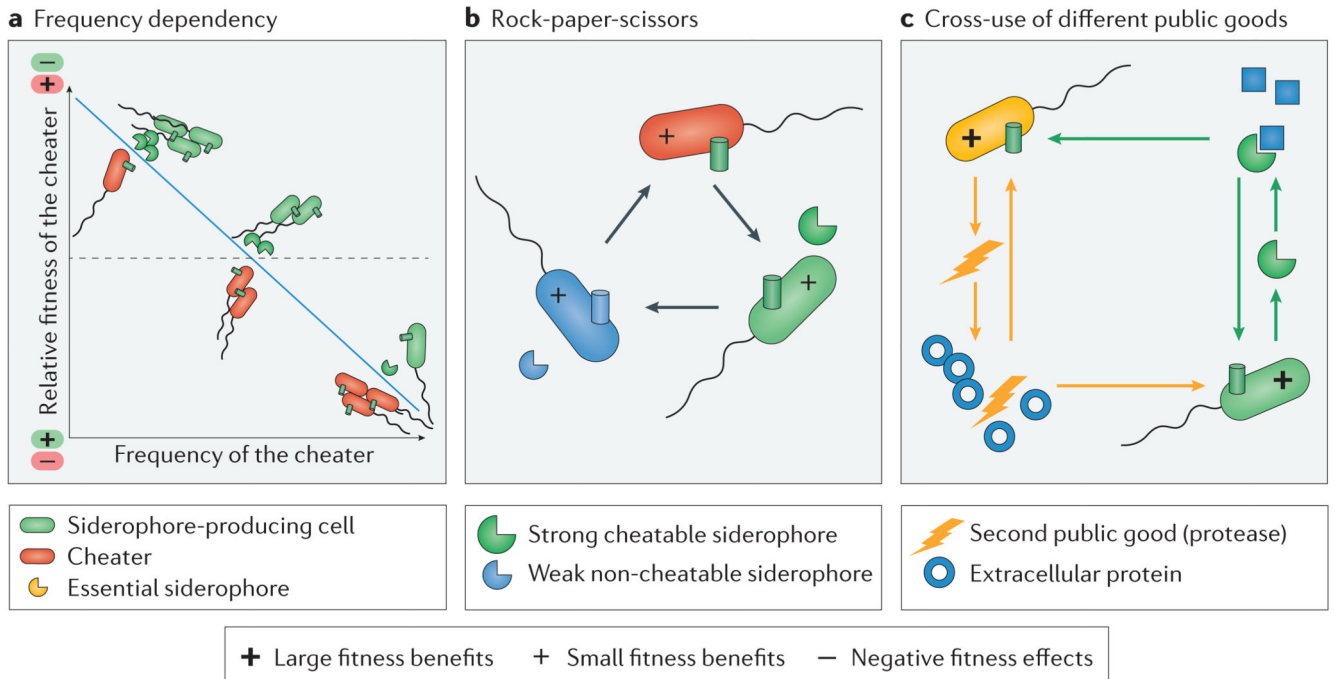
**Figure 3. Siderophores can synergistically increase iron uptake when bacteria live in groups.** The problem of siderophore loss due to diffusion can be reduced when cells live in groups. In this scenario neither the diffusion range of siderophores (yellow) nor the amount of iron brought into solution (orange-red gradient) increases per cell, but the probability of iron-loaded siderophores returning to cells increases, because cells can access each other's siderophores (brown area, double black arrows). Emojis inside cells depict the relative fitness consequences for a cell in the different scenarios. This synergistic effect manifests irrespective of whether the iron is relatively homogeneously distributed (**a, b**, blue-shaded

gradient) or occurs in clumped particles (**c**, **d**, blue circles) in the environment, or whether cells are motile (**a**, **c**) or surface-attached (**b**, **d**).



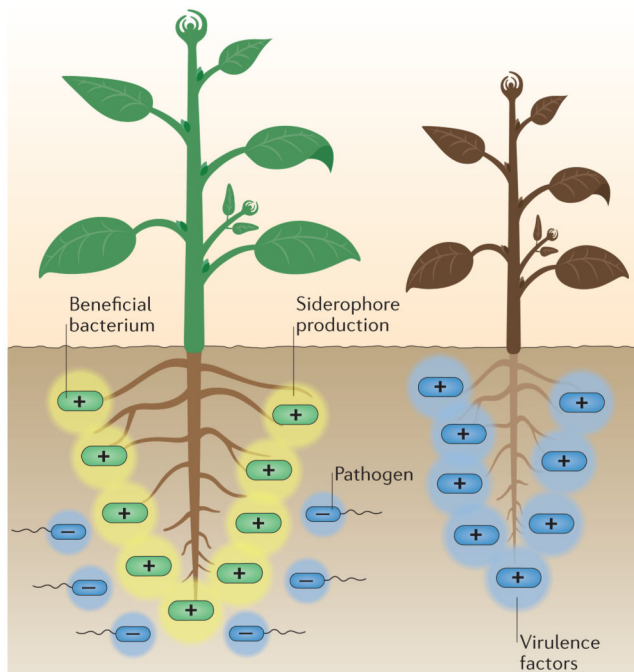
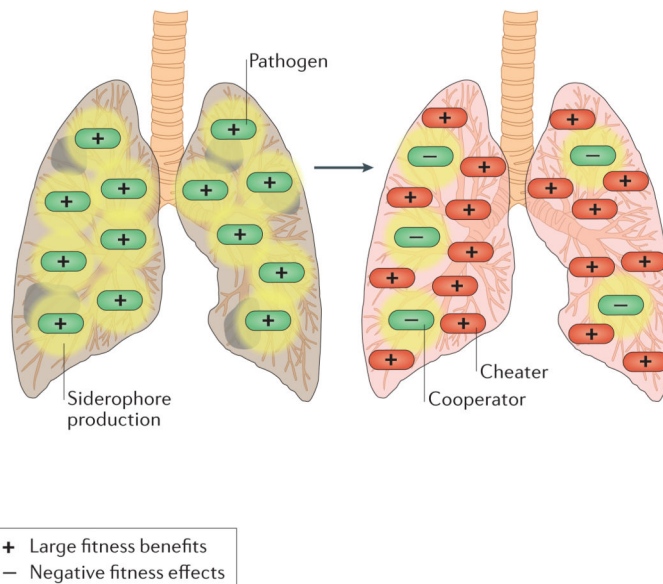
**Figure 4. Siderophore-mediated social interactions.**

The figure shows representative examples of social interactions driven by secreted siderophores (partial circles) that bind insoluble iron (blue squares) and for which specific receptors (cylinders) are required for uptake. Emojis inside cells depict the relative fitness consequences for the interacting partners. **a** | During cooperation in clonal groups, siderophores are mutually shared and accelerate iron uptake through a common siderophore pool. **b** | Cheating happens when siderophore non-producers have the matching receptor for uptake and thereby exploit the common pool of siderophores without contributing to it. **c** | Competition can happen when producers lock iron away from non-producers that lack the matching receptor for heterologous siderophore uptake. **d** | Another competition scenario involves two species that each produce their specific siderophore. The competitive strength of the species is influenced by the amount and kinetics of siderophore production and the iron affinity of their siderophores.



**Figure 5. Siderophores induce eco-evolutionary dynamics in bacterial communities.**

**a** | If siderophores (yellow partial circles) are essential for growth, siderophore producers (green cells) and non-producing cheaters (red cells) can co-exist in a community through negative frequency-dependent selection. **b** | Strains or species with different siderophore strategies can engage in non-transitive community dynamics, and thereby co-exist and foster biodiversity. The example depicts interactions between a cheater (red) outcompeting the siderophore producer, which secretes a compatible siderophore (green). This siderophore producer in turn outcompetes the second siderophore producer (blue), secreting a less potent siderophore. This weaker siderophore producer, however, outcompetes the non-producer because it produces a siderophore for which the non-producer lacks the matching receptor. This scenario leads to a rock-paper-scissors dynamic, in which strains chase each other in circles with no overall winner. **c** | When, in addition to siderophores (green partial circles), a second public good is important for growth (yellow flash, for example, proteases required for extra-cellular protein degradation), specialization could evolve, leading to each strain or species producing only one public good and exchange at the community level. Emojis inside cells depict the relative fitness consequences for the interacting partners.

**a Siderophore-mediated plant protection****b Cooperator-cheater dynamics in infections****Figure 6. Siderophores and their effects on hosts.**

**a** | Siderophores (yellow shaded zones) from beneficial rhizosphere bacteria (green cells) can protect plants from pathogens (blue cells) and their virulence factors (blue shaded zones), and keep the plants healthy (left green plant). In this scenario, siderophore incompatibility between the plant beneficial bacteria and the pathogen is thought to lead to plant protection.

**b** | Siderophores (yellow shaded zones) of pathogenic bacteria (green cells) can function as virulence factors and damage host tissue and promote pathogen growth (left lung). If siderophore non-producers (red cells) occur or de novo evolve in such infections, they can act as cheaters. If these cheaters can spread the induced cooperator-cheater dynamic can potentially lower the virulence of the infection. Emojis inside cells depict the relative fitness consequences for the interacting partners.