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Siderophore cheating and cheating resistance shape competition for iron in soil and freshwater Pseudomonas communities

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All social organisms experience dilemmas between cooperators performing group-beneficial actions and cheats selfishly exploiting these actions. Although bacteria have become model organisms to study social dilemmas in laboratory systems, we know little about their relevance in natural communities. Here, we show that social interactions mediated by a single shareable compound necessary for growth (the iron-scavenging pyoverdine) have important consequences for competitive dynamics in soil and pond communities of Pseudomonas bacteria. We find that pyoverdine non- and low-producers co-occur in many natural communities. While non-producers have genes coding for multiple pyoverdine receptors and are able to exploit compatible heterologous pyoverdines from other community members, producers differ in the pyoverdine types they secrete, offering protection against exploitation from non-producers with incompatible receptors. Our findings indicate that there is both selection for cheating and cheating resistance, which could drive antagonistic co-evolution and diversification in natural bacterial communities.

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While microbes have become model organisms to study the evolution of cooperation in laboratory settings, we still know little about the role of microbial cooperative interactions in complex natural communities. Perhaps the most common form of microbial cooperation is the secretion of so-called public goods—compounds that are costly to produce but generate benefits for other cells in the vicinity of the producer. Such public goods include matrix components to build up biofilms, enzymes to digest food, biosurfactants for cooperative swarming and iron-scavenging siderophores. Many laboratory studies focused on the problem of cheating, a scenario where mutants that no longer contribute to public goods undermine cooperation by capitalising on the public goods secreted by others. This body of work has become a paradigm for the public-goods dilemma, showing how a trait that is beneficial for the group can be selected against by the spread of selfish individuals. While highly influential as a general proof of social evolution theory, a key open question is whether cheating at the genetic and behavioural level.

Here, we tackle this question by examining the potential for public-goods cooperation and cheating among pseudomonads from natural communities both at the genetic and behavioural level. *Pseudomonas* is a diverse genus of γ-proteobacteria, occupying a wide range of habitats (e.g. soil, aquatic ecosystems and animal hosts). Albeit diverse, many fluorescent pseudomonads share an important trait: they can produce and secrete pyoverdine, a siderophore that scavenges insoluble or host-bound iron from the environment. Laboratory experiments have shown that pyoverdine is a public good that can be shared among cells, and be exploited by cheating mutants. Pyoverdine is a secondary metabolite produced via non-ribosomal peptide synthesis. The molecule consists of a conserved chromophore (making this molecule naturally fluorescent), an acyl side chain linked to the chromophore, and a variable peptide chain (6–12 amino acids). The different *Pseudomonas* strains often produce slightly different pyoverdines, varying in the length and the composition of the peptide chain. Moreover, while pyoverdine and its cognate receptor are typically specific, strains can also have less specific and/or several different receptors allowing the uptake of heterologous pyoverdines. This pyoverdine-receptor diversity could facilitate different types of social interactions among co-occurring species. For instance, pyoverdine-producing strains could exploit each other’s pyoverdines. Alternatively, pyoverdine non-producers could gain a foothold by exploiting foreign pyoverdines (i.e. could act as cheats). Moreover, some strains might produce exclusive pyoverdine types, which remain inaccessible for competing non-isogenic strains because they lack a matching receptor—a scenario that could confer resistance to cheating.

Although it has long been conjectured that the above-mentioned interactions could be important drivers of ecological and evolutionary dynamics in microbial communities, there is a lack of studies that have systematically examined siderophore-mediated social interactions, and the resulting fitness consequences among natural isolates in replicated communities. To date, the most comprehensive evolutionary study on siderophore-mediated interactions used a combination of whole-genome sequencing and phenotype screening to show that many marine *Vibrio* strains have lost the siderophore-synthesis cluster, but kept the receptor for uptake. While this genomic pattern is compatible with the idea of siderophore non-producers being cheats, a direct demonstration of cheating during one-to-one strain competition is missing. Here, we build on the work by Cordero et al. and demonstrate that: (a) pyoverdine non-producers co-occur with producers in soil and freshwater communities of *Pseudomonas*; (b) non-producers can exploit siderophores of certain community
members but not of others; (c) exploitation can lead to cheating, where non-producers gain a relative fitness advantage over producers in direct competition; (d) certain pyoverdines inhibit rather than promote the growth of non-producers; and (e) the patterns of cheating and growth inhibition can be explained by receptor compatibility and pyoverdine differences between strains at the molecular level. Taken together, our findings suggest that selection for cheating and resistance to cheating could spur antagonistic co-evolution and strain diversification in natural bacterial communities.

**Results**

**Natural strains vary in their pyoverdine production levels.** We isolated 320 putative *Pseudomonas* strains from a total of eight soil and eight pond communities. For all isolates, we sequenced the *rpoD* gene (a commonly used phylogenetic marker for this genus) to confirm that 315 isolates are pseudomonads. To investigate whether pyoverdine non-producers co-exist with producers in the same community, we measured the pyoverdine production profile of all 315 isolates in iron-limited casamino acids (CAA) medium. This assay revealed that strains producing no or residual amounts of pyoverdine (i.e., producing less than 5% compared to the laboratory reference strains listed in Supplementary Table 1) occurred in 14 of the 16 communities, with an overall abundance of 8.9% in both pond and soil communities. We further found that pyoverdine production is a continuous trait, with the production level ranging from zero to very high amounts (Figs. 1a, b). Variation in pyoverdine production levels was high in all communities (mean coefficient of variation, CV ± SE for soil communities: 61.8 ± 7.3%; for pond communities: 49.6 ± 3.4%; Supplementary Fig. 1a), indicating that non-, low- and high-producer strains typically co-exist.

**Pyoverdine is important for growth under iron limitation.** Compatible with the view that pyoverdine is important for iron acquisition in these pseudomonads, we observed a significant positive correlation between the isolates’ pyoverdine production levels and their growth in CAA medium, where iron was either bound to human apo-transferrin (Fig. 1c; linear mixed model (LMM): $t_{298} = 12.0, p < 0.001$) or the synthetic chelator 2,2′-dipyridyl (Supplementary Fig. 2a; LMM: $t_{298} = 19.67$, $p < 0.001$). To confirm that it is indeed the amount of pyoverdine that determines growth in iron-limited CAA and not the inability of certain strains to consume the provided nutrients, we further cultured the isolates in CAA supplemented with 40 μM FeCl₃. This experiment revealed substantial growth for all strains, showing that all environmental isolates can use CAA as a nutrient source (Fig. 1d, paired $t$-test comparing growth of each strain in iron-limited vs. iron-rich CAA: $t_{134} = -39.40, p < 0.001$).

**Linking pyoverdine production profiles to phylogeny.** To explore the relationship between phylogeny and pyoverdine production, we constructed maximum-likelihood phylogenetic trees based on partial *rpoD* gene sequences. We constructed separate trees for soil and pond habitats, and mapped pyoverdine production profiles of strains onto the trees (Fig. 2). The absolute phylogenetic diversity was significantly higher for pond than for soil communities (median ± (1st quartile | 3rd quartile) of Faith’s phylogenetic diversity for soil communities: 1.43 ± (0.97 | 1.63); for pond communities: 2.49 ± (2.22 | 2.63); Mann–Whitney U-test: $W = 59$, $p = 0.003$). Conversely, the CV of phylogenetic
distance within community (a relative measure of diversity) did not differ between soil and pond, and was high for both habitats (mean CV ± SE for soil: 67.9 ± 6.6%; for pond: 58.9 ± 2.6%; t-test: \( t_{14} = 1.26, p = 0.227 \); Supplementary Fig. 1b). These analyses show that pseudomonads generally live in diverse, multi-strain communities.

We then examined whether there is a phylogenetic signal for pyoverdine production (i.e. whether closely related strains show similar production profiles). For this analysis, we calculated Blomberg’s \( K \) for each community, whereby, \( K = 1 \) indicates a phylogenetic signal as expected under the Brownian motion model of character evolution, and \( K \)-values close to zero stand for weak phylogenetic signals. While we found a moderate phylogenetic signal for pond communities (mean ± SE across communities: \( K = 0.254 ± 0.072 \)), there was no phylogenetic signal for pyoverdine production in soil communities (\( K = 0.009 ± 0.005 \); t-test for difference between habitats: \( t_{14} = 3.37, p = 0.005 \)). These analyses highlight that pyoverdine production levels are not phylogenetically fixed, but highly vary even among closely related strains.

### Supernatants with pyoverdine affect growth of receivers.

We carried out a supernatant assay to test whether pyoverdine secreted by producers stimulates or inhibits the growth of community members producing no or little pyoverdine. From each community, we chose three strains producing no or low amounts of pyoverdine (henceforth called receivers) and fed them with the supernatant of three random strains (henceforth called donors), which produced higher amounts of pyoverdine than the receivers. Thus, each receiver was fed with pyoverdine-containing supernatants from three donors. This supernatant assay revealed that donor supernatants could both stimulate and inhibit the growth of receivers, with the effects varying on a continuum from complete inhibition to strong stimulation (Fig. 3a). The average effect of supernatants on growth did not significantly differ between soil and pond isolates (linear mixed model, LMM: \( t_{14} = 0.731, p = 0.477 \)). Interestingly, the growth effect correlated positively with the phylogenetic relatedness (based on \( rpoD \) sequences) between receivers and donors in soil but not in pond communities (Fig. 3b; LMM for pond: \( t_{14} = 0.69, p = 0.492 \); for soil: \( t_{14} = 5.32, p < 0.001 \)). This result indicates that receivers from soil communities tended to be stimulated by more closely related strains. In pond communities, on the other hand, relatedness between receiver and donor pairs was generally lower than in soil (the highest receiver–donor \( rpoD \) identity was 92.5%, compared to 100% among soil isolates), and receivers were often stimulated by more distantly related donors (Fig. 3b).

The supernatant assays further revealed a significant donor effect (ANOVA: \( F_{47,76} = 3.90, p < 0.0001 \)) and a receiver effect approaching significance (ANOVA: \( F_{43,80} = 1.49, p = 0.062 \)). This means that supernatants of donors generally had consistent (either stimulating, neutral or inhibiting) effects on multiple receivers, and that receivers were often similarly affected by foreign supernatant regardless of the identity of the donor.

**Pyoverdine is responsible for the observed growth effects.** We then examined whether the above-reported stimulatory and inhibitory effects are indeed triggered by pyoverdine. To test this, we first randomly picked eight non-producers (four isolates were complete non-producers, i.e. relative pyoverdine production was indistinguishable from background fluorescence; and four isolates produced residual amounts of pyoverdine, i.e. 1.4–3.1% of laboratory reference strains; Table 1) from different soil and pond communities. We then grew each non-producer under iron-limited conditions with or without a purified pyoverdine from a producer from the same community, which showed a stimulatory effect on the non-producer in the supernatant assay (Fig. 3). We found a perfect match between the two assays (Fig. 4): all the eight pyoverdines isolated from strains previously shown to be stimulatory significantly promoted the growth of the non-producers. This suggests that these eight non-producers possess receptors to exploit the supplemented heterologous pyoverdines.

In a next assay, we fed the same eight non-producers with a purified pyoverdine from producers, which showed a neutral (two cases) or inhibitory (six cases) effect in the supernatant assay.
In five cases results were consistent across the two assays: pyoverdines isolated from strains previously shown to be inhibitory significantly compromised the growth of the non-producers. Although the match was not perfect in the remaining three cases (Fig. 4, community a and b: neutral effect in the supernatant assay vs. inhibition in the pyoverdine cross-feeding assay; community A: inhibitory effect in the supernatant assay vs. neutral effect in the pyoverdine cross-feeding assay), pyoverdine never had a stimulatory effect. These findings strongly suggest that these non-producers lack receptors for the uptake of this second batch of pyoverdines. In this scenario, growth suppression can arise because incompatible pyoverdines lock away iron in the media, thereby further reducing the availability of this essential element for non-producers.

Cheating and cheating resistance in direct competition. The above findings indicate that it is mainly pyoverdine that drives the interaction patterns between our natural isolates under iron limitation. Consequently, we sought to understand how pyoverdine-mediated growth effects (ranging from stimulation to inhibition) impact the competitive abilities of strains. Accordingly, we carried out 16 direct pairwise competition assays where we mixed the eight non-producers with either their stimulating or non-stimulating (neutral or inhibitory) competing strains, we integrated a constitutively expressed, fitness-neutral, mCherry marker into the chromosome of the non-producer in iron-limited medium. In contrast, these growth patterns indicate that it is a handicap to be a pyoverdine non-producer in iron-limited medium. When grown as monocultures, the non-producers grew significantly worse than the producers (Fig. 5a, paired t-test for non-producers vs. stimulating producers: \( t_7 = -3.87, p = 0.006 \); non-producers vs. inhibiting producers: \( t_7 = -9.64, p < 0.001 \)). These growth patterns indicate that it is a handicap to be a pyoverdine non-producer in iron-limited medium. In contrast, fitness patterns reversed in direct competition for four strain pairs, where the non-producers could significantly outcompete their stimulating producers (Fig. 5b). This finding strongly
suggests that non-producers can act as cheats by successfully exploiting the pyoverdine secreted by producers, thereby gaining a relative fitness advantage. However, our competition assays also revealed that the ability to use a heterologous pyoverdine is not necessarily enough to gain a relative fitness advantage, as evidenced by the four cases where the non-producers lost in competition against producers secreting a compatible pyoverdine (Fig. 5b). Finally, we found that the non-producers performed worse and were strongly outcompeted in co-cultures with producers secreting an incompatible pyoverdine (Fig. 5b).

The genetic basis of pyoverdine-mediated social interactions.

The results from the pyoverdine cross-feeding and competition assays suggest that: (a) the growth-stimulating and non-stimulating pseudomonads from the same community produce different pyoverdines; (b) the non-producers have receptors for heterologous pyoverdine uptake; and (c) the pyoverdine receptors of the non-producers are more similar to the receptors of the stimulating than the non-stimulating producers. To test these hypotheses, we sequenced the whole genome of the 24 strains used in the pyoverdine cross-feeding and competition assays (Figs. 4 and 5).

We first compared the organisation of the pyoverdine locus of each strain to that of previously characterised pseudomonads.34, 17, 30 We found that each producer has a single complete pyoverdine locus, consisting of genes encoding the iron-starvation sigma factor pvdS, the pyoverdine-synthesis machinery (i.e. the non-ribosomal peptide synthesis assembly line), the export elements required for secretion, and a receptor (i.e. a fpvA homologue) for uptake (Table 1). In contrast, the four complete non-producers have a highly truncated pyoverdine cluster, where large genomic regions coding for the synthesis machinery are missing. In contrast, pvdS and the receptor gene are still present in these strains (Table 1). The four strains producing residual amounts of pyoverdine, meanwhile, all have a complete pyoverdine cluster (Table 1). The reason why the latter strains are unable to produce wild type amounts of pyoverdine must therefore reside in alterations of regulatory elements, as was found to be the case for evolved P. aeruginosa pyoverdine non-producers31, 32. Because the exact pyoverdine regulon is unknown for our natural isolates, it was impossible to directly test this hypothesis. In summary, our assembly analysis revealed that there are two types of non-producers: structural non-producers with a truncated pyoverdine locus and silent non-producers with a complete, yet largely inactive locus.

To investigate hypothesis (a), we used the non-ribosomal peptide synthesis assembly line identified in the producers to predict the peptide sequence of the pyoverdine backbone. These analyses indicate that the stimulating and the non-stimulating producers, from the same communities, produce structurally different pyoverdines (Table 1), which means that each non-producer was indeed confronted with two different types of pyoverdines in our assays. When focusing on pyoverdine uptake, we found that all the non-producers have multiple homologues of the fpvA receptor in their genome (Table 2). This finding supports hypothesis (b), as it shows that the non-producers (but also the producers) seem to be equipped for taking up heterologous pyoverdines. Given the many fpvA homologues, the direct testing of hypothesis (c) becomes difficult, as we do not know which homologue is actually used to take up the specific pyoverdine provided in our assays. Nonetheless, we performed two comparisons that serve as proxies for testing hypothesis (c). In the first of these, we compared the sequence similarity of FpvA encoded in the producer’s pyoverdine locus to any FpvA homologues in the non-producer. In the second comparison, we analysed the similarity of the FpvA sequence encoded in the pyoverdine locus (in residual non-producers) or close to its remains (in complete non-producers) to any of the FpvA homologues in the producers. Both comparisons were in support of hypothesis (c): receptor similarities were higher between the non-producers and their corresponding stimulating producers than between the non-producers and the non-stimulating producers (comparison 1: respective FpvA similarities (mean ± SE) were 0.58 ± 0.05 vs. 0.40 ± 0.01, paired t-test: \( t_7 = 3.61, p = 0.009 \); comparison 2: respective FpvA similarities were 0.79 ± 0.05 vs. 0.48 ± 0.11, paired t-test: \( t_7 = 3.15, p = 0.016 \); Table 2).

Discussion

Cheating is characterised by individuals exploiting the benefits of cooperative acts performed by others.33 This phenomenon has been extensively studied in microbial laboratory systems in the context of fruiting body34, 35 and biofilm6, 36 formation, group defence strategies37, 38, swarming motility5, 39 enzyme3, 4, toxin40 and siderophore2, 12, 41–43 production. Although social interactions and cheating seem to cover all aspects of microbial life, their role in natural microbial communities remains largely unclear (apart from fruiting body formation)44–47. Our study tackled this gap in knowledge and shows that social interactions mediated by shareable iron-scavenging pyoverdines can have important consequences for strain–to-strain interactions in phylogenetically diverse natural soil and pond Pseudomonas communities. In particular, we found that: (a) strains that produce no or low amounts of pyoverdine commonly occur in natural communities, although iron is a key growth-limiting factor for pseudomonads in natural habitats11, (b) there are two types of non-producers, which both likely evolved from ancestral producers: structural non-producers with a truncated pyoverdine locus and silent non-producers with a complete, yet largely inactive locus; (c) the non-producers possess multiple pyoverdine receptors, and they are able to capitalise on
pyoverdine produced by other community members; (d) certain non-producers can outcompete producers from the same community in direct competition through pyoverdine exploitation; and (e) some producers secrete incompatible pyoverdines that repress rather than promote the growth of non-producing community members. These results highlight that siderophores play an important, multi-faceted role in shaping social interactions between co-occurring bacterial strains, and are likely to drive diversification and competitive dynamics in natural bacterial communities.

Our study revealed that pyoverdine-mediated social interactions among natural pseudomonads are complex, and not only involve cheating, as observed for laboratory strains, but also include pyoverdine-mediated growth inhibition, and the use of heterologous pyoverdine that results in an absolute but not a relative fitness benefit for non-producers (Figs. 3–5). One reason for this increased complexity is that social interactions in our natural communities occur among phylogenetically diverse strains (Fig. 2), which differ in the pyoverdines they produce and the number and types of receptors they possess (Tables 1–2). For instance, our observation that certain non-producers can exploit and outcompete producers suggests that these non-producers possess a high-affinity receptor for specific heterologous pyoverdines. Conversely, our observation that certain non-producers are inhibited by other heterologous pyoverdines suggests that these non-producers possess incompatible receptors, which excludes them from social interactions, and thus allows producers to privatise pyoverdine and iron uptake. Finally, our finding that some non-producers can exploit heterologous pyoverdines, but do not outcompete the producers, could indicate that these non-producers have receptors with relatively low affinity for these particular pyoverdines, or that non-producers were kept in check by producers through other mechanisms, such as toxin-mediated interference competition.

Another source of complexity in our observation is that pyoverdine production is a continuous and not a binary trait, as it is typically the case in laboratory experiments where knockout mutants are used as non-producers. Genetic work on laboratory _P. aeruginosa_ strains revealed that point mutations in the regulatory gene pvdS can lead to continuous variation in PvdS activity and levels of pyoverdine production. The consequence of this is that not only non-producers can exploit producers, but potentially any strain that produces a lower amount of pyoverdine than its competitor could act as a cheat. Moreover, the continuous nature of the pyoverdine trait could favour facultative cheating, where strains invest to some extent in their own pyoverdine production when growing alone, but switch to the exploitation of heterologous pyoverdines when other strains are nearby. Our genomic analysis supports the idea of strains exhibiting flexible facultative strategies, as most sequenced isolates possess multiple different pyoverdine-receptor homologues (median 4, range 1–19; Table 2). Moreover, strains like those four that had a complete pyoverdine locus, yet only produced residual amounts of pyoverdine could be candidates pursuing facultative strategies: sustain themselves with the little amount of pyoverdine they make when growing alone, but switch to exploitation in co-culture with other producers.

We might now ask what the consequences of the reported social interactions for the long-term evolutionary dynamics in natural communities might be. Previous work proposed that one way to escape cheating is to mutate pyoverdine and receptor types. This evolutionary response of producers could in turn impose selection on non-producers to mutate their receptors accordingly, to acquire new compatible receptors through horizontal gene transfer or to evolve broad-range receptors. These evolutionary adaptations and counter-adaptations could infinitely continue and lead to antagonistic co-evolution generating ever new variants of pyoverdines and receptors. Several of our findings are in line with the scenario of antagonistic co-evolution: we observed a high pyoverdine diversity among producers (i.e. the 16 producers analysed in Table 1 produced 11 different pyoverdine types). Furthermore, we found that many strains possess multiple pyoverdine receptors (Table 2), and non-producers could only use the pyoverdine of certain producers but not of others (Fig. 4). Finally, some producers were consistently resistant against exploitation by multiple non-producers, whereas other producers were particularly vulnerable to exploitation. All these findings together indicate that some strains might be ahead of the evolutionary race, by
Table 2 Numbers and similarities of the FpvA pyoverdine receptors between producers and non-producers

| Community ID | Strain ID | Strain classification    | Number of fpvA homologues | Highest similarity of the FpvA in the producer’s pyoverdine locus to the FpvA homologues in the non-producer | Highest similarity of the FpvA in the non-producer’s pyoverdine locus to the FpvA homologues in the producer |
|--------------|-----------|--------------------------|---------------------------|--------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| soil a       | s3a18     | Residual non-producer    | 2                         | 0.76 0.36 1.00                                                                                   | 0.84                                                                                               |
|             | s3a10     | Stimulating producer     | 1                         | 1.00 – 0.84                                                                                      |                                                                                                   |
|             | s3a12     | Non-stimulating producer | 2                         | – 1.00 0.83                                                                                      |                                                                                                   |
| soil b       | s3b5      | Residual non-producer    | 4                         | 0.44 0.38 1.00                                                                                   | 0.92                                                                                               |
|             | s3b6      | Stimulating producer     | 2                         | 1.00 – 0.92                                                                                      |                                                                                                   |
|             | s3b2      | Non-stimulating producer | 2                         | – 1.00 0.88                                                                                      |                                                                                                   |
| soil f       | s3f7      | Residual non-producer    | 4                         | 0.46 0.41 1.00                                                                                   | 0.89                                                                                               |
|             | s3f10     | Stimulating producer     | 5                         | 1.00 – 0.89                                                                                      |                                                                                                   |
|             | s3f19     | Non-stimulating producer | 11                        | – 1.00 0.23                                                                                      |                                                                                                   |
| soil h       | s3h17     | Complete non-producer    | 3                         | 0.74 0.36 1.00                                                                                   | 0.89                                                                                               |
|             | s3h14     | Stimulating producer     | 4                         | 1.00 – 0.89                                                                                      |                                                                                                   |
|             | s3h9      | Non-stimulating producer | 2                         | – 1.00 0.83                                                                                      |                                                                                                   |
| pond A       | 3A5       | Complete non-producer    | 5                         | 0.50 0.42 1.00                                                                                   | 0.62                                                                                               |
|             | 3A18      | Stimulating producer     | 4                         | 1.00 – 0.62                                                                                      |                                                                                                   |
|             | 3A7       | Non-stimulating producer | 4                         | – 1.00 0.24                                                                                      |                                                                                                   |
| pond E       | 3E20      | Complete non-producer    | 7                         | 0.45 0.37 1.00                                                                                   | 0.84                                                                                               |
|             | 3E19      | Stimulating producer     | 3                         | 1.00 – 0.84                                                                                      |                                                                                                   |
|             | 3E13      | Non-stimulating producer | 19                        | – 1.00 0.24                                                                                      |                                                                                                   |
| pond F       | 3F3       | Residual non-producer    | 10                        | 0.59 0.41 1.00                                                                                   | 0.51                                                                                               |
|             | 3F6       | Stimulating producer     | 2                         | 1.00 – 0.51                                                                                      |                                                                                                   |
|             | 3F5       | Non-stimulating producer | 8                         | – 1.00 0.37                                                                                      |                                                                                                   |
| pond H       | 3H3       | Complete non-producer    | 9                         | 0.69 0.47 1.00                                                                                   | 0.83                                                                                               |
|             | 3H7       | Stimulating producer     | 3                         | 1.00 – 0.83                                                                                      |                                                                                                   |
|             | 3H9       | Non-stimulating producer | 6                         | – 1.00 0.25                                                                                      |                                                                                                   |

either being particularly successful in heterologous pyoverdine exploitation or by being generally resistant to it.

For a complete understanding of the system, we would need to know how the pairwise interactions investigated in our study add up at the community level. While a definite answer is not yet possible, a recent laboratory study examined population dynamics in communities where non-producers simultaneously interacted with one producer secreting a compatible pyoverdine, and another producer secreting an incompatible pyoverdine. This study revealed non-transitive competitive dynamics, where non-producers outcompeted producers with a compatible pyoverdine, but were themselves outcompeted by producers with an incompatible pyoverdine. Overall, strains chased each other in a competitive race with no overall winner, which resulted in the maintenance of biodiversity and stable community composition. Our data now reveal that cheating non-producers and cheating-resistant producers are indeed both present in our communities, which opens the possibility for these biodiversity-promoting mechanisms to operate in natural systems.

How does our work compare to the seminal study by Cordero et al., who showed that siderophore non-producing and producing Vibrio strains co-exist with one another in a marine ecosystem? One important insight from our study is that social interactions between producers and non-producers are not restricted to marine communities, but also occur in soil and freshwater ecosystems, in a completely different taxon. This highlights that siderophore-mediated interactions between taxonomically diverse strains are likely a common feature of microbial communities. Another similarity between the two studies is that there is no strong phylogenetic signal for siderophore production. This means that pyoverdine non- or low producers were not limited to a few specific taxonomic clades, but occurred across the entire phylogenetic tree. This indicates that, as for the Vibrio system, non-producers frequently arise de novo from within producer clades. However, the mechanism by which non-producers evolve differs between the Vibrio and our Pseudomonas system. Particularly, Cordero et al., found discrete phenotypes: strains were either full producers (40%) or non-producers (60%), and these phenotypes correlated well with the presence or absence of the siderophore-synthesis clusters in the genome of these strains. Conversely, in our system non-producers are relatively rare (9%) and come in two different forms: structural non-producers with a truncated pyoverdine locus and silent non-producers with a complete, yet largely inactive locus, producing only residual amounts of pyoverdine. In addition, isolates showed continuous phenotypes, from pyoverdine non- to full production. This demonstrates that most isolates have not lost their pyoverdine-synthesis cluster, and suggests that modifications in regulatory elements might rather be the key determinants of how much pyoverdine a strain is capable to produce.

Another key difference between the two study systems is that structural diversity exists for pyoverdine (Table 1), whereas the
**Vibrio** siderophores (aerobactin and vibriobactin) come in a single molecular form\(^\text{3,5}\). Because pyoverdine diversity could select for receptor diversity\(^\text{3,5}\), successful cheating is then not so much about having a receptor per se, but rather about having a matching receptor (Table 2). We can think of two scenarios of how matching receptors can be acquired. First, if non-producers evolve de novo from producers, then they inherently possess the matching receptor of the producer they originated from. This route to exploitation might commonly apply in spatially structured habitats where de novo non-producers can rely on closely related producers staying in close vicinity. Indeed, our supramutant assays suggest that pyoverdine-mediated growth stimulation preferentially occurs among closely related strains in soil, a highly structured environment (Fig. 3b). Second, non-producers could acquire matching receptors through horizontal gene transfer. This route to exploitation might preferentially occur in habitats with low spatial structure, where strains readily mix and closely related producers are not necessarily nearby. This scenario indeed seems to apply to our pond communities, living in a fairly unstructured habitat, where strains readily mix and closely related producers are not primarily occurring among more distantly related strains (Fig. 3b).

In conclusion, our findings demonstrate that pyoverdine-mediated cheating and competition for iron are prevalent among natural *Pseudomonas* isolates and have important fitness consequences. Because iron scavenging via siderophores is ubiquitous among bacterial taxa in iron-limited habitats\(^\text{11,55}\), we propose that siderophore-mediated social interactions are important in many ecosystems, and are likely involved in shaping strain diversity and community dynamics. We further propose that other microbial social traits might play similar roles. For instance, many bacterial species secrete small signalling molecules (e.g. acyl homoserine lactones, AHLs) for communication and the coordination of group-level activities\(^\text{56}\). It has previously been shown that AHLs are exploitable public goods\(^\text{3,4}\), are structurally diverse and occur in many different species\(^\text{37}\). The complex social interactions uncovered here for siderophores might thus well apply to AHL-based communication systems. Taken together, our study highlights that not only abiotic but also social components need to be considered in order to fully understand microbial community assembly and functioning.

**Methods**

**Sampling and isolation of pseudomonads.** We sampled 16 *Pseudomonas* communities from soil and pond habitats (n = 8 each) located on the campus of the University of Zurich Irchel (47.40° N, 8.54° E), Switzerland. We used the following sampling and isolation protocol, adapted from previous studies\(^\text{48,49}\). For soil sampling, we used a metal soil probe with a 7 mm diameter slot to sample the lower 2 cm, and processed the middle part for strain isolation. For pond sampling, we used a metal soil probe with a 7 mm diameter slot to sample the upper 10 cm of the soil. From the extracted soil cores, we discarded the upper and lower 2 cm, and processed the middle part for strain isolation. For soil sampling, we used a metal soil probe with a 7 mm diameter slot to sample the lower 2 cm, and processed the middle part for strain isolation. For pond sampling, we used a metal soil probe with a 7 mm diameter slot to sample the upper 10 cm of the soil. From the extracted soil cores, we discarded the upper and lower 2 cm, and processed the middle part for strain isolation.

After incubation, we picked 20 random isolates per community and streaked them on Luria-Bertani (LB) agar plates to augment and function. We further propose that other microbial social traits might play similar roles. For instance, many bacterial species secrete small signalling molecules (e.g. acyl homoserine lactones, AHLs) for communication and the coordination of group-level activities\(^\text{56}\). It has previously been shown that AHLs are exploitable public goods\(^\text{3,4}\), are structurally diverse and occur in many different species\(^\text{37}\). The complex social interactions uncovered here for siderophores might thus well apply to AHL-based communication systems. Taken together, our study highlights that not only abiotic but also social components need to be considered in order to fully understand microbial community assembly and functioning.

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**Measurement of growth and pyoverdine production levels.** To evaluate whether natural isolates can produce pyoverdine, we grew all isolates under iron-limited conditions and assessed their pyoverdine production levels. We first grew all isolates in 150 µL LB well plates overnight in a 600 µM FeCl₂ solution distributed in 96-well PCR plates. Plates were sealed with an adhesive film and incubated at 30 °C for 18 h. Following 18 h of incubation at room temperature in the dark, we measured growth (optical density OD at 600 nm) and pyoverdine production levels (relative fluorescence units (RFU) with excitation: 400 nm and emission: 460 nm) with an Infinite M200 Pro microplate reader (Tecan Group Ltd., Switzerland)\(^\text{65}\). We then calculated the relative growth and pyoverdine production for each isolate by dividing its OD\(_{600}\) and RFU by the average respective OD\(_{600}\) and RFU of the reference strains.

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non-parametric spline fits using the R ‘gofit’ package (Supplementary Table 3). From these spline fits, we extracted the integral (area under the curve) as the growth parameter of interest. The integral is a representative growth measure as it combines information on the lag, growth and stationary phase in one single estimate\(^6\). Flow cytometry was carried out on a subset of soil (n = 24) and pond (n = 24) isolates grown in CA medium with 200 µM 2,2'-dipiridyl in a 96-well plate for 24 h. OD\(_{600}\) was subsequently measured in 1:20 microplate reader and samples were subsequently diluted 100× in 0.85% NaCl solution. Cells were fixed with glutaraldehyde (final concentration 2.5%, Sigma-Aldrich, Switzerland) and stained with Sybr Green I (5×10\(^{-3}\)dilution of commercial stock, Invitrogen, USA) for 10 min at room temperature in the dark. Samples were analysed using an InFlux V-GS cell sorter (Becton Dickinson Inc., USA). A blue laser (200 mW, 488 nm) was used for detection of side-scattered (SSC) light and Sybr Green I fluorescence (531 nm). Analysis of flow cytometry data was carried out with an in-house custom software (J. Villiger and J. Perenthaler, University of Zurich, unpublished) and bacterial cells were determined using SSC vs. green fluorescence. These control experiments revealed that no significant correlations between end point OD\(_{600}\) growth integrals and cell counts demonstrating that using end point OD\(_{600}\) is a reliable measure of growth for the natural isolates (Supplementary Fig. 4).

**Supernatant assay.** For each community, we harvested supernatants from three pyoverdine-producing isolates (donors) and fed them to three receiver strains. Receivers were always the three isolates with the lowest pyoverdine production levels in the community (mean ± SE relative pyoverdine production was 0.785 ± 0.056, n = 4). To generate pyoverdine-containing supernatants, we grew isolates in 4 ml CA medium with 200 µM 2,2'-dipiridyl in 14 ml polypropylene round-bottom tubes, shaken (160 rpm) at 28°C. Supernatants were isolated in late exponential phase (OD\(_{600}\) = 0.3–0.5, measured with Tcanc microplate reader), and centrifuged for 2 min at 7,500 rcf (Eppendorf Centrifuge 5804R). We then filter-sterilized supernatants by passing them through a 0.22 µm PES membrane filter and kept them at −20°C. Meanwhile, we grew the receivers in 1 ml LB in 24-well plates for 24 h at static room temperature. Then, 1.5 µl of receiver cultures were added to CA with 200 µM 2,2'-dipiridyl without or with 20 µl of donor supernatant (total cultured in 96-well plates in both conditions). The culture was then filter-sterilized, such that values of ln(w) > 0 or ln(w) < 0 indicate whether non-producers have won or lost the competition, respectively.

**Pyoverdine cross-feeding assay.** To test whether it is pyoverdine that triggers the observed growth effects, we performed cross-feeding assays using eight strain triplets (Table 1). We chose eight receivers, which produced less than 5% of pyoverdine compared to our reference strains. Each receiver originated from a different community (four soil and four pond communities). For each receiver, we picked two pyoverdine producers, which were previously shown to either stimulate or inhibit pyoverdine compared to our reference strains. Each receiver originated from a soil or pond community (Supplementary Table 3). The relative fitness of the non-producer as observed in the cross-feeding assay was evaluated using the R package ‘gofit’. We calculated the relative fitness of the non-producer as w = (a - (1-a))/(a - (1-a)) where a0 and a1 are the initial and final frequency of the non-producer, respectively\(^9\). Subsequently, we log-transformed w\(_0\), such that values of ln(w) > 0 or ln(w) < 0 indicate whether non-producers have won or lost the competition, respectively.

**Genomic DNA isolation for whole-genome sequencing.** The 24 isolates were first grown on LB agar plates and then streaked out on Gould’s S1 agar medium (supplemented with 40 µM FeCl\(_3\)) and grown for two days at 28°C. Single colonies were inoculated into 4 ml LB in 14 ml polypropylene round-bottom tubes and grown overnight at 30°C shaken (200 rpm). Genomic DNA was extracted from 1 ml of cultures using GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Switzerland) according to the manufacturer’s instructions, without RNase treatment. The main changes: we used a separately purchased proteinase K (20 mg/ml; Fermentas), and eluted DNA with EB elution buffer (Qiagen).

**Whole-genome sequencing and bioinformatic analysis.** Library preparation and sequencing of the 24 isolates was done at the Lausanne Genomics Technologies Facility with the Illumina Hiseq 2500 platform (paired end 2×250 bp) in Rapid Run mode. Libraries were constructed using the TrueSeq Nano DNA kit. Reads were filtered with Trimmomatic (Supplementary Table 3) and assembled with SPAdes 3.10.1 (Supplementary Table 3) using default parameters. Identification of putative coding sequences and their annotation were performed by the RAST automated annotation pipeline (Supplementary Table 3). The pyoverdine gene cluster was identified manually by a combination of keyword searches in the annotation and BLASTP searches with genes from the reference database against the genomes of isolates. In order to get gene-family profiles independent of annotation we also inferred an orthology using OrthoFinder (Supplementary Table 3). To compare the FpvA receptor encoded in the pyoverdine locus of the producers to receptors of the non-producers, the amino acid sequences of the FpvA receptor from the pyoverdine locus of the stimulators, and likewise the non-stimulators, were blasted against the genomes of the residual or complete non-producers. In the same way, the FpvA receptor sequence of residual and complete non-producers was compared to stimulating and non-stimulating producers. The best hits of the BLAST searches were then compared. The pyoverdine peptide structure was predicted from the amino acid sequence of non-ribosomal peptide synthetases by using PKS/NRPS annotation website (Supplementary Table 3).

**Statistical analysis.** We used linear and LMM models for statistical data analysis. All statistical tests are two-tailed, and p-values ≤ 0.05 were regarded as significant. Since strains isolated from the same community might not be independent from one another we built community identity as a random factor into our models. For our analysis on donor and receiver effects, we further added the strain ID of the donor as a random factor to our models. Whenever appropriate, we used log-transformed (natural logarithm) data to meet the assumption of normally distributed residuals. For phylogenetic analysis we used the R package APE v3.2 (Supplementary Table 3). Blommers’ K-values were calculated with the phyllogenial function from the R package picante v1.6-2 (Supplementary Table 3). All statistical analyses were carried out using R 3.1.2 program (www.r-project.org).

**Data availability.** The experimental and sequencing data that support the findings of this paper have been deposited in the figshare repository (doi:10.6084/m9.figshare.5125093) and in the European Nucleotide Archive under the study accession number PRJEB21289 (http://www.ebi.ac.uk/ena), respectively.

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Author contributions
E.B. and R.K. designed the study, E.B. isolated the strains and performed the experiments, M.B. and S.W. did the bioinformatic analyses, and all authors analysed the data and wrote the paper.

Additional information
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