Vibrio fischeri siderophore production drives competitive exclusion during dual-species growth

Running title: *V. fischeri* siderophore prevents competitor growth

Michaela J. Eickhoff[1] and Bonnie L. Bassler[1,2]*

[1]Department of Molecular Biology, Princeton University, Princeton, NJ 08540, USA.

[2]Howard Hughes Medical Institute, Chevy Chase, MD 20815, USA.

*For correspondence: E-mail bbassler@princeton.edu; Tel. (+1) 609 258 2857.

Summary

When two or more bacterial species inhabit a shared niche, often, they must compete for limited nutrients. Iron is an essential nutrient that is especially scarce in the marine environment. Bacteria can use the production, release, and re-uptake of siderophores, small molecule iron chelators, to scavenge iron. Siderophores provide fitness advantages to species that employ them by enhancing iron acquisition, and moreover, by denying iron to competitors incapable of using the siderophore-iron complex. Here, we show that cell-free culture fluids from the marine bacterium *Vibrio fischeri* ES114 prevent growth of other vibrio species. Mutagenesis reveals the aerobactin siderophore as the inhibitor. Our analysis reveals a gene, that we name *aerE*, encodes the aerobactin exporter, and LuxT is a transcriptional activator of aerobactin production. In co-culture, under iron-limiting conditions, aerobactin production allows *V. fischeri* ES114 to competitively exclude *Vibrio harveyi*, which does not possess aerobactin production and uptake genes. By contrast, *V. fischeri* ES114 mutants incapable of aerobactin production lose in competition with *V. harveyi*. Introduction of *iutA,* encoding the aerobactin receptor, together with *fhuCDB*, encoding the aerobactin importer are sufficient to convert *V. harveyi* into an “aerobactin cheater”.

Keywords: siderophore, *Vibrio fischeri*, aerobactin, iron, competition, genes, regulator

Introduction

Microbial competition is a driving force in ecosystems in which limited resources exist (reviewed by Hibbing *et al.*, 2010; Foster and Bell, 2012; Stubbendieck and Straight, 2016; Ghoul and Mitri, 2016). Marine bacteria, in particular, must survive in seawater that is frequently depleted for essential nutrients (Bristow *et al.*, 2017). Success in inhabiting heterogeneous and/or nutrient-poor marine environments often relies on exploitation of transient microscale nutrient “hot spots” that are associated with detritus, marine snow particles, and marine phytoplankton and zooplankton (reviewed by Stocker and Seymour, 2012). In such environments, key limiting nutritional resources specify the

particular competitive interactions that take place. Competitive strategies can be indirect, for example, rapid capture of a limiting resource (Pfeiffer *et al.*, 2001; Khare and Tavazoie, 2015), while direct competition strategies include the production of antibiotics or other toxic compounds (Abrudan *et al.*, 2015), the formation of surface-associated biofilms that physically exclude competitors from nutritious territory (Nadell and Bassler, 2011), and the contact-dependent delivery of toxic effector molecules into competitor cells via type VI secretion (Logan *et al.*, 2018).

In marine environments, vibrios are a ubiquitous and abundant bacterial genera (reviewed by Thompson *et al.*, 2004). To explore mechanisms underlying interspecies marine bacterial competition, here, we developed a dual-species co-culture model containing two *Vibrionaceae* species. We focused on two well-studied vibrios, *Vibrio harveyi* and *Vibrio fischeri,* that are known to occur together in nature (Ramesh *et al.*, 1989; Ruby and Lee, 1998). Each species can exist free-living in the ocean and associated with marine animals, including in the case of *V. fischeri*, as a symbiont (McFall-Ngai and Ruby, 1991; Alcaide, 2003). In this initial study, we restricted our analyses to their free-living lifestyles using shaking liquid cultures for co-incubations. Attractive features of the *V. fischeri*-*V. harveyi* co-culture model are that both species grow under identical laboratory conditions, and many tools exist for genetic modifications, allowing us to precisely manipulate each species and quantitatively measure the consequences. Moreover, a variety of endogenous and heterologous reporter genes are available for monitoring each strain in isolation and in combination.

Surprisingly, in our initial attempt to characterize the co-culture system, we found that *V. fischeri* produces and releases an inhibitor that prevents the growth of *V. harveyi*. We discovered that the inhibitor is the siderophore aerobactin. Siderophores are small molecule high-affinity iron chelators that many bacteria produce and secrete to scavenge iron (reviewed by Neilands, 1995). In this case, aerobactin produced by *V. fischeri* is released into the culture medium, it chelates the available iron, and as a consequence, *V. harveyi* cannot grow.

Iron is an essential nutrient for virtually all organisms and, germane to the current work, is required by bacteria as a cofactor for enzymes involved in DNA synthesis, electron transport, and the TCA cycle (Crosa *et al.*, 2004). Iron acquisition presents a

challenge to most bacteria because, despite its abundance, it has poor solubility in the presence of oxygen at physiological pH (Hem, 1972). More extreme is the situation in the marine environment: dissolved iron concentrations in the ocean are typically in pM to nM ranges, below the level required for growth of most bacteria (Rijkenberg *et al.*, 2014). The remarkable finding that atmospherically-transported Saharan dust enables proliferation of vibrio bacteria by delivering dissolved iron to surface marine environments further demonstrates the exquisite scarcity of iron in the ocean (Westrich *et al.*, 2016).

Vibrio species commonly produce one or more siderophores to acquire iron (Thode *et al.*, 2018). *V. harveyi* encodes genes for the biosynthesis of two siderophores, anguibactin and amphi-enterobactin (Naka *et al.*, 2013; Zane *et al.*, 2014). *V. fischeri* siderophore production has been demonstrated (Graf and Ruby, 2000; Cordero *et al.*, 2012), and indeed, our scanning of the *V. fischeri* genome sequence using AntiSMASH, a tool that identifies biosynthetic gene clusters (Medema *et al.*, 2011), revealed biosynthetic genes for one hydroxamate siderophore, aerobactin. Aerobactin biosynthetic genes were first characterized in enteric species (de Lorenzo *et al.*, 1986; de Lorenzo and Neilands, 1986; Bailey *et al.*, 2018), and we use these earlier findings to underpin our analyses of the genes involved in *V. fischeri* siderophore production recognizing that the structure of *V. fischeri* aerobactin could differ from that of other species.

Iron exists in two states, the reduced ferrous (Fe^{2+}) form and the oxidized ferric (Fe^{3+}) form. While ferrous (Fe^{2+}) iron is soluble, ferric (Fe^{3+}) iron primarily exists in insoluble ferric hydroxides. Siderophores have high affinity for ferric (Fe^{3+}) iron, the abundant environmental form of iron at neutral pH in the presence of oxygen (Crosa *et al.*, 2004). In Gram-negative bacteria, once synthesized, siderophore export typically occurs via a membrane-spanning transporter protein (reviewed by Saha *et al.*, 2013). Released siderophores bind to the otherwise unavailable ferric (Fe^{3+}) iron. Subsequently, siderophore-iron (Fe^{3+}) complexes are recognized by specific outer membrane (OM) receptors of the TonB-dependent receptor family, with TonB providing the energy for import across the OM. A periplasmic binding protein shuttles the siderophore-iron (Fe^{3+}) complex to an inner-membrane ABC transporter that delivers

the complex to the cytoplasm. Finally, iron must be released from the siderophore either by reducing iron from the ferric (Fe^{3+}) form to the ferrous (Fe^{2+}) form, promoting its release, or via enzymatic cleavage of the siderophore (reviewed by Payne *et al.*, 2016).

In environments harboring multi-species bacterial communities, siderophores are known to play roles in competition for iron (Weaver and Kolter, 2004; Schiessl *et al.*, 2017; Bruce *et al.*, 2017). Siderophore production enables iron capture and use for the producer while simultaneously denying competitors access to an essential nutrient. When two or more species each produce siderophores, the species that produces the siderophore with the highest affinity for iron can enjoy a competitive advantage (Joshi *et al.*, 2006). However, because siderophores are released from cells, they are considered public goods and are susceptible to exploitation by non-producing cells. Indeed, many bacterial species possess genes for siderophore binding and uptake, but they lack genes for siderophore production. Such siderophore-non-producing bacteria are considered “cheaters”, capable of using heterologous siderophores without expending the energy required for their synthesis (Traxler *et al.*, 2012; Cordero *et al.*, 2012; Leinweber *et al.*, 2017; Butaitė *et al.*, 2017).

Here, we identify the siderophore aerobactin as a small molecule inhibitor secreted by *V. fischeri* that prevents growth of *V. harveyi.* While *V. harveyi* cannot produce or import aerobactin, we identify other vibrio species that are natural aerobactin cheaters: they do not produce aerobactin, but they use it. We identify the *V. fischeri* genes required for production, release, and uptake of aerobactin, revealing AerE as a transporter that is used for aerobactin export. We pinpoint the minimal subset of genes required to convert *V. harveyi* into an aerobactin cheater. We use mutagenesis to identify regulators of aerobactin production, revealing LuxT as a transcriptional activator of *V. fischeri* siderophore biosynthesis genes. We investigate the mechanism underlying natural variation in siderophore production between two *V. fischeri* strains. Finally, we demonstrate that in dual-species co-culture, siderophore production allows *V. fischeri* to establish a niche and exclude growth of its competitor *V. harveyi,* conferring a fitness advantage to *V. fischeri* when grown under iron-limiting conditions.

Results

V. fischeri *ES114 produces a siderophore that prevents growth of other vibrio species*

Marine environments are estimated to have 10^4 to 10^6 prokaryotic cells per milliliter (Whitman *et al.*, 1998), and metagenomic studies have revealed that there exists marked microbial phylogenetic diversity (Sogin *et al.*, 2006; Sunagawa *et al.*, 2015). Vibrios are a ubiquitous and abundant bacterial genera in the marine environment (reviewed by Thompson *et al.*, 2004). How vibrios thrive in multi-species marine environments raises questions concerning cooperation and competition. To explore the requirements for dual-species growth and whether interspecies interactions play roles in promoting or preventing cohabitation, we focused on two well-studied vibrio species, *V. harveyi* and *V. fischeri*. We grew cultures of our wild-type (WT) *V. harveyi* strain, BB120, (called *V. harveyi* from here forward), *V. fischeri* ES114, and *V. fischeri* MJ11 alone and in the presence of 10% (v/v) cell-free culture fluids from the other strains/species. *V. fischeri* ES114 culture fluids prevented *V. harveyi* growth in minimal marine medium, whereas identical preparations from *V. fischeri* MJ11 did not (Fig. 1A). The inability to grow was not specific to *V. harveyi*: *V. fischeri* ES114 culture fluids also prevented growth of *Photobacterium angustum* S14 and *Vibrio cholerae* C6706*,* while no diminishment of growth yield of *Vibrio parahaemolyticus* BB22OP and *Vibrio vulnificus* ATCC 29306 occurred (Fig. 1B). The growth inhibition activity was specific to *V. fischeri* ES114, as culture fluids prepared from all the other tested strains did not inhibit *V. harveyi* growth (Fig. S1).

V. fischeri ES114 culture fluids retained the ability to prevent *V. harveyi* growth following boiling or filtration through a 10,000 MWCO membrane, suggesting that the inhibitor is a small molecule (Fig. S2A). *V. fischeri* ES114 culture fluids only inhibited *V. harveyi* growth when *V. fischeri* ES114 was cultured in minimal marine medium but not when it was grown in rich medium (Fig. S2B). The culture fluids prevented *V. harveyi* growth when isolated from *V. fischeri* ES114 grown to an OD_{600} of approximately 0.7 or higher (Fig. S2C). Finally, the presence of *V. fischeri* ES114 culture fluids prevented the growth but did not kill *V. harveyi.* While below the level of detection by OD_{600} measurements, a small increase in *V. harveyi* cell density could be detected by counting

colony forming units (CFUs) (Fig. S2D). These results indicate that the inhibitory substance produced by *V. fischeri* ES114 accumulates during growth, and moreover, is either regulated by nutrients, that a component present in rich medium masks or destroys the inhibitor, or that *V. harveyi* can overcome inhibition if the exogenously-supplied culture fluids contain additional nutrients.

To identify the inhibitor, we conducted a random transposon mutagenesis screen with the goal of isolating *V. fischeri* ES114 mutants incapable of inhibitor production. Our rationale was that culture fluids from such mutants would not prevent the growth of *V. harveyi*. We isolated 6,720 *V. fischeri* ES114 insertion mutants. *V. harveyi* was grown in the presence of 50% culture fluids from the *V. fischeri* ES114 insertion mutant strains, and bioluminescence was measured as an indicator of *V. harveyi* growth. We also measured the final OD_{600} of each *V. fischeri* ES114 mutant to identify mutants harboring transposon insertions that caused severe growth defects. These mutants were eliminated from the screen because we assumed that culture fluids from such mutants would likely possess low amounts of inhibitor, and thus allow growth of *V. harveyi*. Sixty-five *V. fischeri* ES114 mutants were identified without obvious growth defects and whose culture fluids allowed growth of *V. harveyi* as indicated by light production of 10-fold or more above background (Fig. S3A). False positive hits were eliminated in our follow-up analysis: cell-free cultures fluids from the 65 *V. fischeri* ES114 candidate mutant strains were prepared and tested, this time at 5%, for the ability to prevent *V. harveyi* growth. Seventeen *V. fischeri* ES114 transposon insertion mutants appeared defective in production of the growth-inhibitory substance (Fig. S3B).

The genes harboring transposon insertions in the 17 *V. fischeri* ES114 mutants were identified by arbitrarily-primed PCR and sequencing of the transposon-chromosome junctions (Fig. S3B). One operon, *iucABCD,* encoding the biosynthetic enzymes for the siderophore aerobactin, harbored 5 of the transposon insertions (Fig. 2A). Aerobactin is a hydroxamate siderophore originally identified in the Gram-negative bacterium *Aerobacter aerogenes* (now known as *Enterobacter aerogenes)* (Gibson and Magrath, 1969). Aerobactin production is common to enteric bacteria such as *Escherichia coli, Shigella*, and *Salmonella* spp. (reviewed by Payne *et al.*, 2016). Aerobactin is known to be made by *Vibrio mimicus* (Moon *et al.*, 2004), *Vibrio hollisae*

(Okujo and Yamamoto, 1994; Suzuki *et al.*, 2006), and other marine vibrio isolates, including environmental isolates of *V. fischeri* (Cordero *et al.*, 2012).

We hypothesized that, in minimal marine medium, *V. fischeri* ES114 releases aerobactin. Thus, when its culture fluids are added to *V. harveyi*, iron sequestration prevents *V. harveyi* growth. To test this notion, we deleted the entire *iucABCD* operon from *V. fischeri* ES114. We also deleted only *iucD*, encoding a lysine monooxygenase that functions in the first step of aerobactin biosynthesis (de Lorenzo *et al.*, 1986). Culture fluids from both mutant *V. fischeri* ES114 strains did not prevent *V. harveyi* growth (Fig. 2B). We used the liquid chrome azurol S (CAS) siderophore detection dye assay to measure siderophore in the culture fluids (Schwyn and Neilands, 1987; Payne, 1994). WT *V. fischeri* ES114 culture fluids possessed siderophore whereas fluids from the Δ*iucABCD* and Δ*iucD* mutants did not (Fig. 2C). Also, consistent with our above finding that culture fluids isolated from *V. fischeri* MJ11 do not inhibit *V. harveyi* growth, *V. fischeri* MJ11 culture fluids possessed no detectable siderophore (Fig. 2C). To test if inhibition of *V. harveyi* growth was indeed due to chelation of iron by aerobactin, we added increasing concentrations of iron together with the 10% WT *V. fischeri* ES114 culture fluids to *V. harveyi*. In this experiment, soluble ferrous (Fe^{2+}) iron was provided, and from here forward, we distinguish the form of iron supplied in our experiments as either “ferrous (Fe^{2+}, no siderophore required)” or “ferric (Fe^{3+}, siderophore required)”. Addition of 3 μM or higher ferrous (Fe^{2+}, no siderophore required) iron rescued *V. harveyi* growth (Fig. 2D). We conclude that iron chelation is responsible for the inhibition of *V. harveyi* growth when *V. fischeri* ES114 culture fluids are present. As shown in Fig. S3 and described below, 8 additional *V. fischeri* ES114 genes were identified in the mutagenesis screen that potentially encode components that decrease aerobactin production via regulation of *iucABCD*, reduction in secretion or recycling of aerobactin, or alteration of metabolism to indirectly influence siderophore biosynthesis or activity.

Fur represses aerobactin production in V. fischeri *ES114*

It was curious that growth inhibition of *V. harveyi* occurred only when culture fluids were obtained from *V. fischeri* ES114 grown in minimal marine medium but not in rich

medium (Fig. S2B). We suspected that either *V. fischeri* ES114 does not produce aerobactin when grown in rich medium, and/or sufficient iron is present in rich medium to overcome aerobactin chelation. To explore these possibilities, we quantified transcription of the *V. fischeri* ES114 siderophore biosynthetic genes under the two conditions. There was only low level *iucA* expression in *V. fischeri* ES114 grown in rich medium. In minimal marine medium, *iucA* transcription increased with increasing cell density until late exponential phase. Specifically, at $OD_{600} = 0.7$, *iucA* transcription was 30-fold higher in *V. fischeri* ES114 grown in minimal marine medium than when it was grown to the same OD_{600} in rich medium (Fig. S4). Activation of *iucABCD* expression could occur in minimal marine medium or repression could occur in rich medium. A likely candidate for controlling transcription by a repressive mechanism is Fur (ferric uptake regulator), the major transcriptional regulator of iron transport in Gram-negative bacteria. Under iron-replete conditions, typically, Fur binds ferrous iron (Fe^{2+}, no siderophore required), the complex binds so-called Fur box DNA elements, and transcription is repressed (Bagg and Neilands, 1987). Thus, Fur-regulated genes are derepressed under iron-limiting conditions. Aerobactin production is Fur-regulated in *E. coli* (de Lorenzo *et al.*, 1987), and Fur boxes have been identified neighboring the aerobactin biosynthetic genes in *V. hollisae* (Suzuki *et al.*, 2006). The CAS assay shows that ferrous iron (Fe^{2+}, no siderophore required) concentrations of 2 μM or higher repressed siderophore production in *V. fischeri* ES114, and deletion of *fur* relieved repression (Fig. 3A). When introduced on a plasmid, *mVenus* fused to the *iucABCD* promoter was likewise repressed in response to increasing ferrous iron (Fe^{2+}, no siderophore required) concentration. No repression occurred in the absence of Fur (Fig. 3B). Using a colorimetric ferene dye assay for iron, we found that the concentration of iron in our minimal marine medium is ~0.3 μM (Fig. S5) Thus, in minimal marine medium, limited iron availability coupled with high siderophore production by *V. fischeri* ES114 impair *V. harveyi* growth in the presence of *V. fischeri* ES114 culture fluids. Increased iron availability and/or Fur-mediated repression of siderophore production in *V. fischeri* ES114 allows *V. harveyi* growth.

Identification of activators of aerobactin production in V. fischeri *ES114*

As mentioned, our mutagenesis screen identified transposon insertions in 8 genes in addition to those in the aerobactin biosynthetic operon (Fig. S3B). Three genes, *cca, mnmG,* and *trmE*, are involved in tRNA modification. While we do not understand the connection these genes have to aerobactin production, we speculate they may alter the availability of the required L-lysine substrate. We did not study these three genes further. We constructed in-frame deletions in the 5 remaining genes and examined the effects on aerobactin production. Deletion of 4 of the 5 genes, *luxT, yebK, fre,* and *glpK,* significantly reduced aerobactin production in *V. fischeri* ES114 (Fig. 4A). Consistent with this finding, increased *V. harveyi* growth occurred in the presence of culture fluids prepared from the 4 *V. fischeri* ES114 mutants (Fig. 4B). LuxT, YebK, Fre, and GlpK are therefore activators of aerobactin production in *V. fischeri* ES114. *luxT* and *yebK* encode DNA-binding transcriptional regulators. *fre* and *glpK* encode flavin reductase and glycerol kinase, respectively. Deletion of any of these 4 genes did not alter the growth of *V. fischeri* ES114 (Fig. S6). The fifth gene, *glpF*, encoding the glycerol uptake facilitator protein did not fit the above pattern, as in-frame deletion of *glpF* did not reduce aerobactin production (Fig. 4A,B). *glpF* is located in an operon upstream of *glpK*. We presume that transposon insertion in *glpF* is polar on *glpK*, which explains this result. We were surprised that our mutagenesis screen did not identify *glnD,* as a previous report showed that transposon insertion in *glnD* reduces siderophore production by *V. fischeri* ES114 (Graf and Ruby, 2000). To investigate this discrepancy, we deleted *glnD* from *V. fischeri* ES114. The mutant exhibits only a modest decrease in siderophore production and in *iucA* transcription (Fig. S7A,B, respectively). Culture fluids from the Δ*glnD* mutant strain retain the ability to inhibit *V. harveyi* growth (Fig. S7C). Together, these results explain why *glnD* was not revealed in our screen.

To examine the mechanisms underlying control of aerobactin production by LuxT, YebK, Fre, and GlpK, we introduced a plasmid harboring an *iucA'-mVenus* transcriptional fusion or an *iucA*'-'*mVenus* translational fusion into WT *V. fischeri* ES114 and the four deletion mutant strains. Only deletion of *luxT* significantly decreased *iucABCD* transcription (Fig. 4C), and as a consequence, the corresponding activity of the translational reporter was also reduced (Fig. 4D). The strains carrying deletions in

yebK, fre, and *glpK* did not exhibit altered expression of either reporter (Fig. 4C,D). In the Discussion, we provide hypotheses for the role of each of these genes.

LuxT, a member of the TetR transcription factor family (reviewed by Ramos *et al.*, 2005), was originally identified as a repressor of *luxO* encoding the central response regulator in the *V. harveyi* quorum-sensing (QS) system (Freeman and Bassler, 1999; Lin, Miyamoto, and Meighen, 2000a; Lin, Miyamoto, and Meighen, 2000b). To test if LuxT regulates siderophore production in a QS-dependent manner, we measured siderophore production in *V. fischeri* ES114 Δ*ainS,* Δ*ainR*, and Δ*litR* Δ*luxR* strains encoding a QS autoinducer synthase, QS autoinducer receptor, and the two QS master transcriptional activators, respectively (Engebrecht and Silverman, 1984; Gilson *et al.*, 1995; Fidopiastis *et al.*, 2002; Kimbrough and Stabb, 2013). There was no difference in siderophore production in the mutants compared to WT, indicating that LuxT regulates siderophore production independently of QS (Fig. S8).

We tested whether LuxT control of *iucABCD* transcription is direct. We used a strategy in which we overexpressed *luxT* in WT and Δ*fur E. coli* MG1655 harboring the *iucA'-mVenus* transcriptional reporter plasmid. There was no change in reporter output when *luxT* expression was induced, indicating that either LuxT functions indirectly to control *iucABCD* transcription or LuxT functions together with some other *V. fischeri* ES114 cofactor that is not present in *E. coli* (Fig. S9A). qRT-PCR confirmed that *luxT* was transcribed from the overexpression vector in *E. coli* as there was a 40-fold increase in *luxT* transcript levels in the strain supplied with the arabinose inducer compared to the isogenic uninduced strain (Fig. S9B). *E. coli* Fur repressed *iucA* transcription, confirming its role in aerobactin repression and the validity of our heterologous system (Fig. S9A). Below, we discuss possible mechanisms by which LuxT could regulate *iucABCD*.

Aerobactin cheating: requirements for aerobactin recognition and uptake

In communities, public goods can be exploited by cheaters who acquire advantages through use of the good but who do not pay the energetic cost of goods production. In the context of siderophores, a cheater need only possess genes required for recognition

and uptake of the siderophore-iron (Fe^{3+}) complex. Often, siderophore biosynthetic genes exist within larger gene clusters harboring genes encoding ancillary functions for reception and transport (reviewed by Crosa and Walsh, 2002). Such is the case for *V. fischeri* ES114: *iucABCD* are present in a 9-gene cluster (Fig. 5A). These 9 genes appear to be conserved and co-inherited in aerobactin producing vibrio strains (Cordero *et al.*, 2012). In the cluster, *iutA,* encoding the OM receptor for the aerobactin-iron (Fe^{3+}) complex, is immediately downstream of the *iucABCD* biosynthetic genes (de Lorenzo *et al.*, 1986). The *fhuCDB* genes are upstream of *iucABCD* and encode an inner membrane ABC transporter in which FhuD is a periplasmic binding protein, FhuB is a membrane permease, and FhuC is an ATP-binding protein (Wooldridge *et al.*, 1992). A gene (*VF_A0157)* encoding a major facilitator superfamily (MFS) transporter resides upstream of *fhuCDB* and is oriented in the opposite direction. This gene is called *shiF* in *Shigella,* but no function has been ascribed to it (Forman *et al.*, 2007). Here, we refer to this gene as *aerE* for aerobactin exporter, a function we demonstrate below.

V. parahaemolyticus and *V. vulnificus* grew in the presence of *V. fischeri* ES114 culture fluids (Fig. 1B), but culture fluids prepared from these species did not inhibit *V. harveyi* growth (Fig. S1). Based on these findings, we hypothesize that *V. parahaemolyticus* and *V. vulnificus* are aerobactin cheaters. Support for this idea comes from inspection of genome sequences. The genome of *V. vulnificus* ATCC 29306, the strain used here, has not been sequenced, however the genomes of the related strain *V. vulnificus* YJ016 and of *V. parahaemolyticus* BB22OP (used here) show that neither possesses the *iucABCD* biosynthetic genes. Both species encode *fhuCDB* and *iutA* homologs (Fig. 5A). We predict that the IutA homolog would recognize and import aerobactin across the OM, and the FhuCDB homologs would transport aerobactin across the inner membrane. Previous studies have shown the requirement for these genes in aerobactin uptake in *V. parahaemolyticus* and *V. vulnificus* strains (Funahashi *et al.*, 2003; Tanabe *et al.*, 2005; Funahashi *et al.*, 2009). To verify this activity in our *V. parahaemolyticus* strain, we deleted either *iutA* or *fhuCDB.* Indeed, when *V. fischeri* ES114 culture fluids were administered to a *V. parahaemolyticus* Δ*iutA* or Δ*fhuCDB* strain, no growth of the recipient occurred (Fig. 5B). Moreover, deletion of *iutA* in *V. fischeri* ES114 made the strain incapable of growth in the presence of its own WT

culture fluids. This defect was complemented by *in trans* expression of *iutA* (Fig. 5B). Together, these data demonstrate the necessity IutA and FhuCDB for uptake of extracellular aerobactin in *both* aerobactin producing and aerobactin cheating strains. To further explore the requirements to be an aerobactin cheater, we turned to *V. harveyi*. We reasoned that, because *V. harveyi* does not encode homologs of any of the nine genes in the aerobactin cluster, we could use *V. harveyi* as a tool to identify the minimal components required for cheating. We introduced plasmids into *V. harveyi* encoding subsets of genes from the *V. fischeri* ES114 aerobactin cluster. Expression of either *iutA* or *fhuCDB* was insufficient to rescue *V. harveyi* growth in the presence of *V. fischeri* ES114 culture fluids. However, *V. harveyi* harboring both *iutA* and *fhuCDB* could grow in their presence (Fig. 5C). We conclude that IutA and FhuCDB, encoding, respectively, the siderophore OM receptor and siderophore importer are sufficient to convert a non-aerobactin-producing species into an aerobactin cheater.

AerE is an aerobactin exporter

The protein encoded by the first gene in the *V. fischeri* ES114 aerobactin cluster (Fig. 5A), that we name *aerE,* encodes a MFS transporter. *aerE* homologs are typically present in aerobactin gene clusters but their roles in iron acquisition and utilization, if any, have not been defined. It has been speculated that homologs of *aerE* encode aerobactin exporters, however, the authors noted uncertainty based on the existing data. Specifically, Forman *et al.* (2007) reported that *E. coli* deleted for the *aerE* homolog remained capable of aerobactin secretion, and Genuini *et al.* (2019) showed that fluids from an *E. coli* mutant deleted for the gene possessed less aerobactin than those from WT.

We sought to clearly define the role of AerE. Deletion of *aerE* from *V. fischeri* ES114 did not reduce the relative level of siderophore present in culture fluids as assessed by the CAS assay (Fig. 6A). However, deletion of *aerE* did cause a growth defect in *V. fischeri* ES114 when the strain was grown in minimal marine medium but not when grown in rich medium (Fig. S10A,B, respectively). We interpret these results to mean that, in minimal marine medium, aerobactin is produced and accumulates to a

toxic level in the Δ*aerE* strain. By contrast, in rich medium, because Fur represses siderophore production (Fig. S2B), no toxicity occurs (Fig. S10B). To test for increased intracellular aerobactin in the *V. fischeri* ES114 Δ*aerE* strain, we measured aerobactin levels in cells (i.e., not in culture fluids). Approximately 40-fold more siderophore was in the cytoplasm of the *V. fischeri* ES114 Δ*aerE* mutant than in the WT (Fig. 6B). Deletion of *fhuCDB* or *iutA* encoding the aerobactin importer and OM receptor, respectively, did not alter intracellular aerobactin levels (Fig. 6B). We conclude that *aerE* and its homologs (i.e., *shiF*) encode aerobactin exporters. We can think of two possibilities to explain the finding that culture fluids from the *V. fischeri* ES114 Δ*aerE* mutant possess normal levels of siderophore (Fig. 6A): either aerobactin-toxicity-mediated cell death and, consequently, lysis, causes siderophore release into the mutant culture fluids, or *V. fischeri* ES114 harbors an additional aerobactin export mechanism(s). We assessed cell death using Sytox Green, a stain that only permeates cells with compromised membranes. We did not detect increased lysis of the Δ*aerE V. fischeri* ES114 strain relative to WT *V. fischeri* ES114 (Fig. S11).

Variation between siderophore-producing and non-producing V. fischeri *strains*

V. fischeri ES114 and *V. fischeri* MJ11 share a high level of conservation between their genomes. Specifically, *V. fischeri* MJ11 encodes over 90% of the open-reading frames present in *V. fischeri* ES114, and shared sequences encode proteins with a median amino acid identity of 98.8% (Mandel *et al.*, 2009). The divergence between the two strains raises interesting evolutionary questions and is, furthermore, responsible for establishing host-specificity: *V. fischeri* ES114 is a symbiont of the squid, *Euprymna scolopes,* whereas *V. fischeri* MJ11 is a symbiont of the fish, *Monocentris japonica* (Boettcher and Ruby, 1990; Mandel *et al.*, 2009). The striking variability in siderophore production by *V. fischeri* ES114 and *V. fischeri* MJ11 strains that do and do not produce detectable aerobactin, respectively (Fig. 2C and Pankey *et al.*, 2017), is not due to a lack of aerobactin biosynthetic genes in *V. fischeri* MJ11. Its genome encodes the entire aerobactin cluster, and there are no obvious frameshift or nonsense mutations present that would abrogate function (Fig. S12A).

To assess the possibility that the difference in *V. fischeri* ES114 and *V. fischeri* MJ11 aerobactin phenotypes is due to differences in gene expression, we used qRT-PCR to quantify mRNA levels of *aerE, fhuC, iucA,* and *iutA*. In order to compare transcript levels, qRT-PCR primers were designed to bind DNA sequences that are 100% identical between the two strains. Surprisingly, transcription of all four genes from the non-producing *V. fischeri* MJ11 strain was equal to or higher than the corresponding gene in the producing *V. fischeri* ES114 strain (Fig. S12B). Thus, differences in transcription cannot underpin the two phenotypes. With respect to the encoded proteins, the IucABCD biosynthetic proteins are less similar (mean 96.4% identity) between the two strains than the proteins encoded by the flanking genes (mean 98.6% identity) (Fig. S12A). We hypothesized that the biosynthetic enzymes encoded by *iucABCD* may be more efficient in *V. fischeri* ES114 than in *V. fischeri* MJ11. To test this possibility, we overexpressed the *iucABCD* operon from each strain in a *V. fischeri* ES114 Δ*iucABCD* mutant and measured siderophore production using the CAS assay. Overexpression of *V. fischeri* ES114 *iucABCD* fully restored the Δ*iucABCD* defect. However, overexpression of the *V. fischeri* MJ11 *iucABCD* genes drove only partial restoration of siderophore production (Fig. 7A). In the reciprocal experiment, overexpression of *iucABCD* from *V. fischeri* ES114 drove a marked enhancement in siderophore production by *V. fischeri* MJ11 over that which occurred when its own *iucABCD* genes were overexpressed (Fig. 7B). Thus, the difference in siderophore production between *V. fischeri* ES114 and *V. fischeri* MJ11 does not stem from differences in transcriptional regulation, rather, the difference apparently arises at the protein level, perhaps due to differences in post-transcriptional regulation, biosynthetic enzymatic activity, or protein stability.

Aerobactin production confers a fitness advantage to V. fischeri *ES114 in co-culture with* V. harveyi *when iron is limiting*

The competitive exclusion principle states that if two species occupy the same ecological niche, the species that multiplies most rapidly, even if only by a small margin, will displace the other species, driving it to extinction given sufficient time (Hardin,

1960). In bacteria, exclusion of the less fit strain can be followed using long-term co-culture growth experiments (Veldkamp and Jannasch, 1972; Bruger and Waters, 2016; Sexton and Schuster, 2017). With our new understanding that aerobactin is an inhibitor of *V. harveyi* growth under iron-limiting conditions, we sought to test its role in dual-species co-culture and whether its presence or absence alters the outcome in competitions between *V. fischeri* ES114 and *V. harveyi*.

Growth rates of each species were first analyzed individually. In our minimal marine medium, *V. fischeri* ES114 and *V. harveyi* have similar growth rates, but *V. harveyi* grows to an overall higher final cell density (Fig. S13A). In an attempt to mimic natural marine environments in which siderophores could play roles in competition, iron was depleted from the medium by treatment with Chelex 100 resin. This process made it so that iron was undetectable in the medium as measured by a colorimetric ferene dye assay (Fig. S5). The limit of detection was approximately 0.02 μM. Next, ferric (Fe^{3+}, siderophore required) iron was added back to the treated medium at 10 μM to provide an iron source that can be accessed by aerobactin and enable significant growth of WT *V. fischeri* ES114. We call this iron-depleted medium. WT *V. fischeri* ES114 grew to 6-fold higher final cell density than *V. harveyi* in the iron-depleted medium. The *V. fischeri* ES114 Δ*iucABCD* mutant did not exhibit higher growth capacity than *V. harveyi* in the iron-depleted medium (Fig. S13B). Therefore, aerobactin is required for growth of *V. fischeri* ES114 to high cell density in iron-depleted medium. *V. harveyi* harbors genes enabling production of two siderophores. Nonetheless, growth of *V. harveyi* was negligible in the iron-depleted medium as 6-fold lower OD_{600} was achieved than in minimal marine medium (compare red lines in Fig. S13A,B). The inability of siderophores to promote *V. harveyi* growth under iron-depleted conditions is likely due to QS repression of siderophore gene expression which restricts siderophore production to low cell density (Lilley and Bassler, 2000). Indeed, culture fluids from WT *V. harveyi* possess 18-fold less siderophore activity than culture fluids from WT *V. fischeri* ES114, as detected by the CAS assay (Fig. S14).

We competed *V. harveyi* against either WT or Δ*iucABCD V. fischeri* ES114 under four different growth conditions. In each case, the two species were combined in a 1:1 ratio, inoculated into the growth medium, and allowed to grow for 24 h. Fitness was

measured by plating and counting CFUs of each species at the start and the end of the co-culture. The *V. fischeri* ES114 and *V. harveyi* colony morphologies are distinct, so they are easily distinguished on agar plates. Additionally, on agar plates, *V. harveyi* produces light and *V. fischeri* ES114 does not, aiding in species discrimination. The selection rate constants were calculated, the preferred analysis method for competitions under starvation conditions (Travisano and Lenski, 1996; Lenski, 2019). A value of 1 indicates that *V. fischeri* ES114 increased in cell density about 1 natural log more than *V. harveyi*. A value of 0 indicates that the two species increased equally in cell density. A value of -1 means that *V. harveyi* increased about 1 natural log more than *V. fischeri* ES114.

In minimal marine medium, *V. harveyi* outcompeted both WT and Δ*iucABCD V. fischeri* ES114 (Fig. 8). This result can be explained by the fact that *V. harveyi* grows to a higher cell density than *V. fischeri* ES114 in minimal marine medium (Fig. S13A). Apparently, aerobactin production plays no role under this condition because iron is sufficiently abundant for both species to grow without the need to produce a siderophore (Fig. S13A). Additionally, when *V. fischeri* ES114 was grown in monoculture in this condition, it did not activate siderophore production until it achieved an OD_{600} of 0.7, about 6 h into growth (Figs. S2C and S4) indicating that by the time *V. fischeri* does produce aerobactin, the growth window in which aerobactin can suppress *V. harveyi* doublings has passed. CAS assays confirmed that *V. fischeri* ES114 does indeed produce siderophore in our competitive setup (Fig. S15). Next, when competed in minimal marine medium supplemented with 10% (v/v) WT *V. fischeri* ES114 culture fluids, both WT and Δ*iucABCD V. fischeri* ES114 out-competed *V. harveyi.* Both *V. fischeri* strains increased by at least 4 natural logs more than *V. harveyi* (Fig. 8). No growth of *V. harveyi* was detected in either competition presumably because the aerobactin in the culture fluids had sequestered the iron. Both *V. fischeri* ES114 strains grew because irrespective of whether the strain produced (WT) or did not produce (Δ*iucABCD*) aerobactin, the siderophore supplied in the exogenous culture fluids could be used for iron acquisition. However, when competed in minimal marine medium supplemented with culture fluids from the Δ*iucABCD V. fischeri* ES114 strain, neither *V. fischeri* ES114 strain showed a growth advantage over *V. harveyi* (Fig. 8). This result

shows that extracellular aerobactin is key for conferring the growth advantage to *V. fischeri* ES114 under our conditions. Finally, when competed in the iron-depleted medium, WT *V. fischeri* ES114 exhibited a significant fitness advantage over *V. harveyi,* increasing by about 2.3 natural logs more than its *V. harveyi* competitor. However, the Δ*iucABCD* mutant *V. fischeri* ES114 strain lost the competition: *V. harveyi* increased about 2.7 natural logs more than the *V. fischeri* Δ*iucABCD* strain (Fig. 8). Therefore, when iron is limiting, as it is in natural marine environments, aerobactin production confers a competitive advantage to *V. fischeri* ES114 over *V. harveyi.* Presumably, in the ocean, the ability of *V. fischeri* ES114 to successfully outcompete other non-cheater, non-aerobactin producing bacteria depends on the release of aerobactin for iron acquisition.

Discussion

When two bacterial species coexist and both species require the same limiting nutrient(s) to grow, competition is favored. The bacterial species that grows most rapidly can drive competitive exclusion of the other species. Different competitive strategies have evolved. In this study, we identified one strategy that can be used by the marine bacterium *V. fischeri* ES114. By producing and secreting the siderophore aerobactin, *V. fischeri* ES114 improves its ability to acquire iron and, therefore, reproduce, while simultaneously denying iron to a competing species, inhibiting its growth. Indeed, aerobactin production alone is sufficient to dictate the fate of *V. fischeri* ES114 when in competition with another vibrio species under iron-depleted conditions.

Siderophores, because they are secreted, are considered public goods, products that are costly for an individual cell to produce, but that can provide benefits to members of a community. Public goods producing bacteria are vulnerable to cheaters, bacteria that benefit from using public goods without paying the metabolic cost of producing them. As shown here and elsewhere, bacterial cheaters that possess genes for siderophore recognition and uptake exist. With respect to aerobactin, the IutA OM receptor and the FhuCDB importer represent the minimal components required to cheat. To become a cheater, an aerobactin producing strain could lose the *iucABCD*

biosynthetic genes. Alternatively, the genes encoding IutA and FhuCDB could be acquired by horizontal gene transfer. Horizontal transfer is known to have distributed aerobactin genes across multiple vibrio phylogenetic lineages (Thode *et al.*, 2018). Siderophore cheating is common in vibrios, and the number of siderophore receptors encoded in a vibrio genome typically exceeds the number of siderophores an individual strain can produce. For example, one strain of *Vibrio tasmaniensis* encodes 7 different siderophore receptor genes, but it only harbors the biosynthetic genes to produce 1 siderophore. A strain of *Vibrio nereis* encodes 6 receptors, but it cannot produce any siderophores (Thode *et al.*, 2018). These examples emphasize the pressure on vibrios to scavenge iron, and are presumably relevant to interspecies interactions that occur in the ocean.

Prior to the present work, *V. fischeri* ES114 was known to produce a siderophore (Graf and Ruby, 2000), however the genes involved in siderophore biosynthesis and uptake had not been characterized. *V. fischeri* ES114 possesses a 9-gene cluster harboring aerobactin biosynthesis, recognition, and import genes. Here, we assign a function to the first gene in the cluster, *aerE,* by showing it encodes a MFS transporter that exports aerobactin. Not surprisingly, *aerE* is conserved in aerobactin producing vibrio strains (Fig. S16A,B and Cordero *et al.*, 2012), because, to profit from siderophore production, a cell must secrete the siderophore so it can perform its extracellular iron-scavenging function. Interestingly, *aerE* is also conserved in aerobactin cheating vibrio strains. While there are exceptions, most aerobactin-cheating vibrios possess a cluster that contains *aerE, fhuCDB*, and *iutA*, all residing in their usual relative positions, however, the biosynthetic *iucABCD* genes are replaced by *iutR,* encoding a GntR family transcriptional regulator (Fig. S16C and Cordero *et al.*, 2012). In *V. vulnificus* M2799, IutR is a repressor of *iutA* (Tanabe *et al.*, 2005).

Given that *aerE* sits in the most distal position in the aerobactin gene cluster, it is reasonable to assume that horizontal transfer of a cassette lacking *aerE* but including the *fhu* and *iutA* genes could have occurred. However, that is not what most vibrio aerobactin cheater genomes show. We hypothesize that acquisition of *aerE* by aerobactin cheater strains provides two possible advantages over acquisition of the minimal *fhuCDB* and *iutA* cheater gene set. First, possession of AerE makes an

591 aerobactin cheater immune to cytoplasmic aerobactin toxicity. We say this based on our
592 assessment of the growth defect displayed by the *V. fischeri* ES114 Δ*aerE* mutant (Fig.
593 S10A). Second, possession of AerE may enable aerobactin recycling by cheater vibrios,
594 fostering higher overall iron acquisition and, in turn, a superior growth advantage during
595 competitive situations.

596 We do not yet understand how *V. fischeri* ES114, as an aerobactin producer,
597 thrives in low-iron environments containing aerobactin cheaters. We note that *V. fischeri*
598 ES114 possesses homologs of *peuA* (*VF_A0191*) and *desA* (*VF_A0784*) encoding
599 putative receptors for the siderophores enterobactin and deferoxamine B, respectively
600 (Tanabe *et al.*, 2011; Cordero *et al.*, 2012; Tanabe *et al.*, 2014). It is possible that *V.*
601 *fischeri* ES114 switches between siderophore-producing and siderophore-cheating
602 based on iron availability and whether or not other vibrios are present that can supply
603 siderophores (aerobactin, enterobactin, or deferoxamine B). Indeed, mechanisms to
604 down-regulate production of siderophores that act as public goods are predicted to
605 evolve in the presence of competitors, whereas siderophores that are privatized and
606 cannot be exploited by competitors are upregulated (Niehus *et al.*, 2017).

607 Regarding possession of aerobactin genes, our growth curve analyses show a
608 shorter lag phase for *V. fischeri* ES114 mutants deficient in siderophore production,
609 secretion, and import, than for WT *V. fischeri* ES114 likely due to the energetic cost to
610 WT *V. fischeri* ES114 of producing and using these components (Fig. S10A,B). The fact
611 that *V. fischeri* maintains these functions, despite the cost, further supports the notion
612 that they play a crucial role in survival. Aerobactin production by *V. fischeri* ES114 could
613 be especially relevant during colonization of its symbiotic host. Microarray analyses
614 revealed that genes for *V. fischeri* siderophore production are upregulated ~5-fold in the
615 *E. scolopes* host where *V. fischeri* ES114 exists in monoculture (McFall-Ngai and Ruby,
616 1991; Wier *et al.*, 2010). Presumably *V. fischeri* ES114 must produce aerobactin in the
617 host as cheating using one of its other siderophore receptors is not a feasible strategy.

618 Here we also identified four activators of aerobactin production in *V. fischeri*
619 ES114: Fre, YebK, LuxT, and GlpK (Fig. 4A,B) and we speculate on their potential
620 roles. First, flavin reductase, encoded by *fre*, reduces flavin using NAD(P)H (Spyrou *et*
621 *al.*, 1991). Siderophore recycling and iron release often occur via reduction of the bound

622 ferric (Fe^{3+}) iron to the soluble ferrous (Fe^{2+}) form for which siderophores have low
623 affinity. Indeed, reduced flavin can transfer electrons to promote the reduction of ferric
624 (Fe^{3+}) iron when it is bound to a siderophore (Coves and Fontecave, 1993; reviewed by
625 Fontecave *et al.*, 1994; Schröder *et al.*, 2003). Perhaps, in *V. fischeri* ES114, Fre
626 participates in such a manner to recycle aerobactin, driving an increase in extracellular
627 siderophore production as detected by the CAS assay. Next, YebK, is a transcriptional
628 regulator with no known function. Unexpectedly, while YebK promotes aerobactin
629 production, it is not via transcriptional activation of *iucABCD* (Fig. 4A,C). YebK is a
630 member of the MurR/RpiR family, and it has homology to HexR in *Pseudomonas putida.*
631 In *P. putida,* HexR is a repressor of the *hex* regulon encoding enzymes required for
632 carbohydrate metabolism by the Entner-Doudoroff pathway. HexR also represses the
633 genes encoding glucokinase and glyceraldehyde-3-phosphate dehydrogenase (Hager
634 *et al.*, 2000; del Castillo *et al.*, 2008). Evidence exists that HexR detects oxidative stress
635 (Kim *et al.*, 2008; Kim and Park, 2014). If YebK functions analogously to HexR, YebK
636 could participate in metabolic pathways to increase the substrates required for
637 aerobactin biosynthesis or to promote its recycling. Additionally, if YebK does indeed
638 detect oxidative stress, it could provide a regulatory mechanism to control iron
639 acquisition during oxidative stress conditions. It could be beneficial to repress iron
640 uptake under oxidative stress because ferrous (Fe^{2+}) iron reacts with hydrogen peroxide
641 in the Fenton reaction to generate harmful hydroxyl radicals that damage DNA (Imlay *et*
642 *al.*, 1988).
643 Next is LuxT, a TetR family transcriptional regulator that activates *iucABCD*
644 transcription. In *V. parahaemolyticus,* the LuxT homolog SwrT activates lateral flagella
645 (*laf)* genes required for swarming motility by an indirect mechanism: SwrT represses
646 *swrZ,* encoding a GntR-type transcriptional regulator that, in turn, represses *laf* (Jaques
647 and McCarter, 2006). *V. fischeri* has a *swrZ* homolog that is a candidate to encode the
648 component that acts between LuxT and *iucABCD*. Finally, GlpK: glycerol kinase
649 catalyzes the first step in glycerol metabolism (reviewed by Lin, 1976). In our mutant
650 collection, the connection between GlpK and aerobactin production is the most difficult
651 to rationalize. The minimal marine medium used in our experiments has glycerol as the
652 carbon source and amino acids are also present. While highly speculative, we suspect

653 that *glpK* mutation demands that *V. fischeri* ES114 use amino acids, rather than
654 glycerol, for growth, altering metabolic pathways that decrease the availability of a
655 substrate required for aerobactin production.

656 Finally, by identifying aerobactin as a growth inhibitory molecule for *V. harveyi*
657 and eliminating its production in *V. fischeri* ES114, we have established a convenient
658 new co-culture model system for studying two well-characterized vibrios. Moreover,
659 both vibrio species produce a variety of public goods including extracellular proteases,
660 chitinases, and QS autoinducers, all of which can be monitored in real time. This co-
661 culture system, in which either or both species can be genetically manipulated, provides
662 a route to the quantitative investigation of both competitive and cooperative interspecies
663 interactions that occur in nature.

664

665 **Experimental Procedures**

666

667 *Bacterial strains and culture conditions*

668

669 *V. fischeri* strains were derived from the *E. scolopes* light organ isolate *V. fischeri*
670 ES114 (Boettcher and Ruby, 1990) and the *M. japonica* isolate MJ11 (Mandel *et al.*,
671 2009). All *V. harveyi* strains were derivatives of *V. harveyi* BB120 (BAA-1116) (Bassler
672 *et al.*, 1997). *V. parahaemolyticus* strains were derived from *V. parahaemolyticus*
673 BB22OP (also known as LM5312) (McCarter, 1998). Strains are listed in Table S1. *E.*
674 *coli* S17-1 λ*pir* was used for cloning, and *E. coli* MG1655 was used for heterologous
675 gene expression. Vibrio strains were grown aerobically with shaking at 30°C in either
676 "rich" Luria-Marine (LM) medium or "minimal marine" Autoinducer Bioassay (AB)
677 medium containing 0.4% vitamin-free casamino acids (Difco) (Greenberg *et al.*, 1979;
678 Bassler *et al.*, 1994). *E. coli* strains were grown aerobically with shaking in LB medium
679 at either 37°C or 30°C. Unless otherwise indicated, erythromycin, chloramphenicol,
680 kanamycin, ampicillin, and polymyxin B were added to final concentrations of 5 μg mL^{-1},
681 10 μg mL^{-1}, 100 μg mL^{-1}, 100 μg mL^{-1}, and 50 μg mL^{-1}, respectively. The plasmid
682 pEVS170 was maintained in *E. coli* by growth in BHI medium (Difco) supplemented with
683 erythromycin at a concentration of 150 μg mL^{-1}. Induction from the P_{tac} promoter was

performed with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), and induction of the P_{BAD} promoter was with 0.2% (v/v) arabinose. Plasmids were transformed into *E. coli* by electroporation using a Bio-Rad Micro Pulser. Plasmids were introduced into vibrios by conjugation with *E. coli* S17-1 λ*pir* on LB plates. *V. fischeri* exconjugants were selected on agar containing ampicillin, and *V. harveyi* and *V. parahaemolyticus* exconjugants were selected on agar with polymxyin B. CFU assessment was performed using serial dilutions and plating.

DNA manipulation and mutant construction

PCR reactions relied on either KOD Hot Start DNA Polymerase (Sigma) or iProof DNA Polymerase (Bio-Rad). Oligonucleotides were obtained from Integrated DNA Technologies and are listed in Table S2. All cloning was performed using isothermal DNA assembly (IDA) (Gibson *et al.*, 2009) using Gibson Assembly Master Mix (New England Biolabs). Plasmids used in this study are listed in Table S3. Sequencing of plasmid DNA and PCR products was conducted at Genewiz. Vibrio chromosomal mutations were generated using the suicide vector pRE112 (Edwards *et al.*, 1998) containing the counter-selectable sucrose marker *sacB* as previously described (Chaparian *et al.*, 2016). Briefly, 1 kb regions of DNA flanking the gene(s) of interest were cloned in tandem into pRE112 and transformed into *E. coli* S17-1 λ*pir*. Following conjugation with the recipient vibrio strain, colonies that had undergone the single crossover recombination event were selected by growth on agar plates containing chloramphenicol. The second crossover event was selected by plating these strains on LM agar plates containing 15% sucrose. Colonies were screened for chloramphenicol sensitivity, and the chromosomal alterations were confirmed by PCR. The *E. coli* Δ*fur* allele came from the Keio Collection (Baba *et al.*, 2006) and was transferred into *E. coli* MG1655 by phage P1*vir* transduction (Silhavy *et al.*, 1984). Expression of the p*iutA* and p*fhuCDBiutA* constructs from their native promoters was accomplished by including approximately 300 bp of upstream DNA in the cloned product. In the case of p*fhuCDBiutA,* the insert was amplified from the *V. fischeri* ES114 Δ*iucABCD* strain.

Cell-free culture fluid preparations and assays

Unless otherwise specified, cell-free culture fluids were isolated as follows. Overnight cultures of the donor *V. fischeri* or other vibrio strains grown in rich medium were pelleted via centrifugation at 21,100 x g (Eppendorf 5424) and resuspended in minimal marine medium. Fresh minimal marine medium (5 mL) was inoculated with the washed cells, normalizing each culture to a starting OD_{600} = 0.01 as measured by a DU800 spectrophotometer (Beckman Coulter). Following growth for 16 h, the OD_{600} was measured and the cells were pelleted by centrifugation at 2,808 x g for 15 min at 4°C (Eppendorf 5810 R). The clarified supernatants were collected and filtered through 0.22 µm filter units (MilliporeSigma™ SLGP033RB). To assay for growth inhibition of *V. harveyi* or other vibrio species, the cell-free culture fluids were aliquotted into clear-bottom 96-well plates (Corning) in quadruplicate technical replicates at 10% (v/v) of the total 200 µL volume. The remainder of the volume was supplied from a 1:1000 dilution of the recipient vibrio species that had been grown overnight in rich medium and washed and resuspended in minimal marine medium. Provision of 10% (v/v) sterile minimal marine medium was used as the no addition control. The plates were covered with gas-permeable sealing membranes (Breathe-Easy) and incubated with shaking at 30°C for 16 h. Growth of the recipient strains was monitored by measurement of OD_{600} using an Envision 2103 Multilabel Reader (Perkin Elmer). When growth inhibition was assayed over time, 25 mL cultures were used. Samples of culture fluids (1 mL) were collected every 2 h and OD_{600} measurements were made. To characterize the inhibitor substance, size exclusion examination of the cell-free culture fluids was performed using 10,000 MWCO Amicon Ultra-15 Centrifugal Filter Units (UFC901024).

V. fischeri *ES114 transposon mutagenesis screen*

A library of *V. fischeri* ES114 transposon mutants was generated as previously described (Lyell *et al.*, 2008). Briefly, the mini-Tn*5* delivery vector pEVS170 was conjugated into *V. fischeri* ES114 using triparental mating with *E. coli* CC118λ*pir* containing the helper plasmid pEVS104 (Stabb and Ruby, 2002). Following incubation

overnight at 30°C, *V. fischeri* ES114 colonies harboring transposon insertions were selected by growth at room temperature on LM agar plates containing erythromycin. Isolated mutant colonies were arrayed into 96-well plates containing rich medium with erythromycin and covered with gas permeable sealing membranes. Following overnight growth at 30°C with shaking, 1 µL of each mutant culture was inoculated into a new 96-well plate containing 200 µL of minimal marine medium. The original plates were stored at -80°C for later access to mutants of interest. The newly-inoculated plates were covered with gas permeable sealing membranes and incubated for 16 h at 30°C with shaking. The plates were subjected to centrifugation at 2,808 x g for 15 min (Eppendorf 5810 R). The resulting culture fluids were supplied at 50% (v/v) to the recipient *V. harveyi* strain by transferring 100 µL to wells of new 96-well plates containing 100 µL of a 1:1000 dilution of a *V. harveyi* culture that had been grown overnight and washed in minimal marine medium. For simplification purposes, we did not filter the remaining mutant *V. fischeri* ES114 cells out of the culture fluids, however, polymyxin B was added to the cultures to prevent further growth of *V. fischeri* ES114 while allowing *V. harveyi* growth. After incubation for 16 h with shaking at 30°C, *V. harveyi* bioluminescence was assessed for each well as a proxy for growth using an Envision 2103 Multilabel Reader. Values were normalized by dividing each well by the average bioluminescence of the entire plate. *V. fischeri* ES114 makes almost no bioluminescence under laboratory conditions so the presence of any residual cells did not contribute to the bioluminescence reading. The insertion sites of the transposons of interest were identified using arbitrarily-primed PCR and sequencing as previously described (O'Toole *et al.*, 1999; Brennan *et al.*, 2013).

Chrome azurol S siderophore detection assay

The CAS liquid assay (Schwyn and Neilands, 1987) was used to measure siderophore activity in cell-free culture fluids from different vibrio strains. The CAS assay solution was prepared as previously described (Payne, 1994). 0.5 mL of CAS assay solution was added to 0.5 mL of cell-free culture fluids prepared from vibrio strains grown in minimal marine medium. After mixing, 10 µL of shuttle solution (0.2 M 5-sulfosalicylic

777 acid) was added and the samples were mixed. Samples were incubated at room
778 temperature for 30 min, after which A_{630} measurements were taken using a DU800
779 spectrophotometer. Minimal marine medium was used as the blank. Siderophore units
780 were calculated as [A_{630}(medium control) – A_{630}(culture fluid)]/A_{630}(medium control) x
781 100 (Payne, 1994). Values were divided by the OD_{600} of the cultures from which the
782 culture fluids were isolated to calculate siderophore units relative to cell density. To
783 measure intracellular siderophore levels, 50 mL cultures were grown in minimal marine
784 medium as described above. After 8 h, the cells were pelleted by centrifugation at 2,808
785 x g for 15 min at 4°C, concentrating and normalizing each to OD_{600} = 40 in 600 µL. After
786 3 washes with 600 µL minimal marine medium, the cells were lysed by boiling for 15
787 min. Cell debris was removed from the lysates by centrifugation for 2 min at 21,100 x g.
788 500 µL of lysate was used in the CAS assay. Because the *V. fischeri* aerobactin
789 structure is unknown, we do not possess the pure compound to generate a standard
790 curve. Thus, concentrations of aerobactin cannot be quantified in our CAS assays, so
791 we restrict our analyses to comparisons of relative levels within each experiment.
792
793 *Quantitative real-time PCR analysis*
794
795 Cultures of *V. fischeri* grown overnight in rich medium were washed in minimal marine
796 medium as above and diluted to an OD_{600} = 0.005 in 25 mL of fresh minimal marine or
797 rich medium. RNA was harvested every 2 h over growth from three independent
798 cultures using the RNeasy mini kit (Qiagen #4104) and OD_{600} was measured at each
799 timepoint. The RNA samples were treated in two sequential DNase reactions with the
800 TURBO DNA-free Kit (ThermoFisher, AM1907). cDNA was generated as described (Tu
801 and Bassler, 2007) using SuperScript III Reverse Transcriptase (ThermoFisher,
802 18080085) with 1 µg of harvested RNA per sample. Real-time PCR was performed in
803 384-well reaction plates using a QuantStudio 6 Flex Real-Time PCR detection system
804 (ThermoFisher) and PerfeCTa SYBR Green FastMix (Quanta, 95074) as previously
805 described (Tu and Bassler, 2007). Reactions (10 µL) were analyzed in quadruplicate
806 technical replicates. Data were analyzed by a comparative $\Delta\Delta C_T$ method in which the
807 relative amount of the indicated transcript was normalized to the internal *hfq* control

gene. qRT-PCR primers are listed in Table S2.

Fluorescent reporter and growth curve assays

To measure *iucABCD* expression, transcriptional and translational reporter fusions to the fluorescent protein mVenus were constructed. For the transcriptional reporter, a region of approximately 300 bp upstream of *iucA* encompassing the promoter was cloned upstream of *mVenus* translated from a consensus ribosome-binding site. The translational reporter included the same 300 bp promoter fragment, but *mVenus* was cloned in frame following the DNA encoding the first thirteen amino acids of lucA. Primers used for reporter construction are listed in Table S2. Reporter activity was measured using a BioTek Synergy Neo2 Multi-Mode reader (BioTek, Winooski, VT, USA). Specifically, overnight cultures of *V. fischeri* ES114 grown in rich medium were washed as above and inoculated into minimal marine medium containing chloramphenicol, normalizing each culture to a starting OD_{600} of 0.005. 150 µL were transferred to a clear-bottom 96-well plate in quadruplicate technical replicates. 50 µL of mineral oil (Sigma) was added to prevent evaporation. In the plate reader, the cultures were grown with constant shaking at 30°C. Both OD_{600} and fluorescence (excitation 515 nm/emission 528 nm) were measured every 15 min for 24 h. For normalization purposes, relative fluorescence values (mVenus/OD_{600}) are reported for each strain at OD_{600} = 0.4. Growth curve assays were similarly conducted: vibrio cultures were grown in clear-bottom 96-well plates (Corning) as above in the indicated growth medium. The plates were incubated with constant shaking at 30°C, and OD_{600} was measured every 15 min for 24 h. To measure p*iucA'-mVenus* transcriptional reporter activity in *E. coli,* recombinant strains were sub-cultured 1:1000 in 1 mL of LB, and grown 6 h with shaking at 30° C. The cells were pelleted by centrifugation and resuspended in sterile PBS prior to fluorescence and OD_{600} measurements using a BioTek Synergy Neo2 Multi-Mode reader. RNA was collected at the 6 h timepoint for qRT-PCR analysis as described above. Lysis was assayed using Sytox Green (ThermoFisher) in which 1 µM dye was added to cultures grown for 16 h in minimal marine medium. Fluorescence (excitation 504 nm/emission 523 nm) and OD_{600} were measured using a BioTek

Synergy Neo2 Multi-Mode reader.

Competition experiments

An iron-depleted growth condition was established for competition experiments by treating 10X minimal marine medium (lacking $MgSO_4$) three times with Chelex 100 resin in batch (Bio-Rad). $MgSO_4$ was left out of the 10X minimal marine medium because Chelex 100 can bind Mg^{2+} ions. 5 g of resin were added to 100 mL of the 10X solution and incubated with stirring for 1 h. The solution was decanted from the resin, and the procedure was repeated two more times. Iron depletion was monitored using a ferene colorimetric dye assay as previously described (Folsom *et al.*, 2014). Briefly, 100 µL of iron-detection reagent was added to 1 mL of medium, and the mixture was incubated for 30 min at room temperature. Purified Milli-Q water (Sigma) was treated identically and used as the blank. Iron concentrations were determined by measuring absorbance at 593 nm (A_{593}) using a DU800 spectrophotometer followed by comparison to a standard curve generated using an iron standard (Sigma). After three Chelex 100 treatments, iron levels were below the level of detection of the ferene assay, approximately 0.02 µM. To make 100 mL of iron-depleted medium, 10 mL of Chelex 100-treated 10X minimal marine medium was combined with 1.23 g $MgSO_4$ and purified Milli-Q water was added to a volume of 100 mL. A trace metals solution lacking iron (Cold Spring Harbor Protocols) was added at 1X and the medium was filtered through a 0.22 µm filter. 10 µM ferric (Fe^{3+}, siderophore required) citrate was added to provide a source of iron that is accessible to siderophore. Following overnight growth in rich medium, 500 µL of five *V. fischeri* and five *V. harveyi* cultures were pelleted by centrifugation (21,100 x g for 2 min), washed twice with iron-depleted medium, and resuspended in iron-depleted medium. The *V. fischeri* and *V. harveyi* suspensions were combined at a 1:1 ratio in 1 mL of the indicated growth medium to a total OD_{600} of 0.005 (0.0025 OD_{600} *V. fischeri* and 0.0025 OD_{600} *V. harveyi*). A portion of this starting culture was collected for serial dilution and plating on agar plates to assess CFUs. Next, 150 µL of each co-culture were transferred to a 96-well plate. 50 µL of mineral oil was added to prevent evaporation. The cultures were grown with shaking for 24 h at 30°C. Serial dilutions and

plating on agar plates were performed to measure the final cell numbers of both species. *V. fischeri* and *V. harveyi* colonies were distinguished by differences in colony morphology and bioluminescence emission. Selection rate constants, *r*, for *V. fischeri* were calculated as a measure of relative performance by subtracting the Malthusian parameter of *V. harveyi* (m_{Vh}) from the Malthusian parameter of *V. fischeri* (m_{Vf}). The Malthusian parameter is calculated as $m_i = \ln(x_1/x_0)$ for competitor *i,* where x_0 and x_1 are the cell densities (CFU mL^{-1}) at the start (x_0) and the end (x_1) of the 24 h growth period. The selection rate constant of *V. fischeri* is calculated as $r = m_{Vf} - m_{Vh}$.

Bioinformatic analyses

The *V. fischeri* ES114 genome was scanned for siderophore biosynthetic genes using AntiSMASH with relaxed strictness (Medema *et al.*, 2011). Bioinformatic searches for homologs of genes in the *V. fischeri* ES114 aerobactin gene cluster were performed using exhaustive BLASTx searches with an expect threshold of 10. The amino acid sequences of the identified homologs were compared in Geneious by pairwise ClustalW alignment. Similarities between homologs are reported as either % pairwise identity in amino acid sequence or % pairwise positive (BLSM62).

Statistical methods

All statistical analyses were performed using GraphPad Prism software in which unpaired two-tailed *t* tests were performed comparing the means of two groups, as indicated in the figure legends. Error bars correspond to standard deviations of the means.

Acknowledgements

We thank members of the Bassler laboratory, Dr. Ned Wingreen, and Dr. Mohamed Donia for insightful discussions and suggestions. This work was supported by the Howard Hughes Medical Institute, National Institutes of Health (NIH) Grant 5R37GM065859, and National Science Foundation Grant MCB-1713731 (to B.L.B). M.E. was supported by NIH graduate training grant NIGMS T32GM007388.

Conflict of interest

The authors declare that they have no conflict of interest.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

MJE and BLB designed all experiments. MJE constructed all strains, conducted all experiments, and analyzed the data. BLB analyzed data and initiated and supervised the project. MJE and BLB wrote the manuscript.

References

Abrudan, M.I., Smakman, F., Grimbergen, A.J., Westhoff, S., Miller, E.L., van Wezel, G.P., and Rozen, D.E. (2015) Socially mediated induction and suppression of antibiosis during bacterial coexistence. *Proc Natl Acad Sci USA* **112**: 11054–11059.

Alcaide, E. (2003) Numerical taxonomy of Vibrionaceae isolated from cultured amberjack (Seriola dumerili) and surrounding water. *Curr Microbiol* **46**: 184–189.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., *et al.* (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular Systems Biology* **2**: 2006.0008.

Bagg, A., and Neilands, J.B. (1987) Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in Escherichia coli. *Biochemistry* **26**: 5471–5477.

Bailey, D.C., Alexander, E., Rice, M.R., Drake, E.J., Mydy, L.S., Aldrich, C.C., and Gulick, A.M. (2018) Structural and functional delineation of aerobactin biosynthesis in hypervirulent Klebsiella pneumoniae. *J Biol Chem* **293**: 7841–7852.

Bassler, B.L., Greenberg, E.P., and Stevens, A.M. (1997) Cross-species induction of luminescence in the quorum-sensing bacterium Vibrio harveyi. *J Bacteriol* **179**: 4043–4045.

Bassler, B.L., Wright, M., and Silverman, M.R. (1994) Multiple signalling systems controlling expression of luminescence in Vibrio harveyi: sequence and function of genes encoding a second sensory pathway. *Molecular Microbiology* **13**: 273–286.

Boettcher, K.J., and Ruby, E.G. (1990) Depressed light emission by symbiotic Vibrio fischeri of the sepiolid squid Euprymna scolopes. *J Bacteriol* **172**: 3701–3706.

Brennan, C.A., Mandel, M.J., Gyllborg, M.C., Thomasgard, K.A., and Ruby, E.G. (2013) Genetic determinants of swimming motility in the squid light-organ symbiont Vibrio fischeri. *MicrobiologyOpen* **2**: 576–594.

Bristow, L.A., Mohr, W., Ahmerkamp, S., and Kuypers, M.M.M. (2017) Nutrients that limit growth in the ocean. *Curr Biol* **27**: R474–R478.

Bruce, J.B., Cooper, G.A., Chabas, H., West, S.A., and Griffin, A.S. (2017) Cheating and resistance to cheating in natural populations of the bacterium Pseudomonas fluorescens. *Evolution* **71**: 2484–2495.

Bruger, E.L., and Waters, C.M. (2016) Bacterial Quorum Sensing Stabilizes Cooperation by Optimizing Growth Strategies. *Appl Environ Microbiol* **82**: 6498–6506.

Butaitė, E., Baumgartner, M., Wyder, S., and Kümmerli, R. (2017) Siderophore cheating

and cheating resistance shape competition for iron in soil and freshwater Pseudomonas communities. *Nat Commun* **8**: 414–12.

Chaparian, R.R., Olney, S.G., Hustmyer, C.M., Rowe-Magnus, D.A., and van Kessel, J.C. (2016) Integration host factor and LuxR synergistically bind DNA to coactivate quorum-sensing genes in Vibrio harveyi. *Molecular Microbiology* **101**: 823–840.

Cordero, O.X., Ventouras, L.A., DeLong, E.F., and Polz, M.F. (2012) Public good dynamics drive evolution of iron acquisition strategies in natural bacterioplankton populations. *Proc Natl Acad Sci USA* **109**: 20059–20064.

Coves, J., and Fontecave, M. (1993) Reduction and mobilization of iron by a NAD(P)H:flavin oxidoreductase from Escherichia coli. *Eur J Biochem* **211**: 635–641.

Crosa, J.H., and Walsh, C.T. (2002) Genetics and Assembly Line Enzymology of Siderophore Biosynthesis in Bacteria. *Microbiol Mol Biol Rev* **66**: 223–249.

Crosa, J.H., Mey, A.R., and Payne, S.M. (2004) Iron Transport in Bacteria. *ASM Press*.

de Lorenzo, V., and Neilands, J.B. (1986) Characterization of iucA and iucC genes of the aerobactin system of plasmid ColV-K30 in Escherichia coli. *J Bacteriol* **167**: 350–355.

de Lorenzo, V., Bindereif, A., Paw, B.H., and Neilands, J.B. (1986) Aerobactin biosynthesis and transport genes of plasmid ColV-K30 in Escherichia coli K-12. *J Bacteriol* **165**: 570–578.

de Lorenzo, V., Wee, S., Herrero, M., and Neilands, J.B. (1987) Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (fur) repressor. *J Bacteriol* **169**: 2624–2630.

del Castillo, T., Duque, E., and Ramos, J.L. (2008) A set of activators and repressors control peripheral glucose pathways in Pseudomonas putida to yield a common central intermediate. *J Bacteriol* **190**: 2331–2339.

Edwards, R.A., Keller, L.H., and Schifferli, D.M. (1998) Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. *Gene* **207**: 149–157.

Engebrecht, J., and Silverman, M. (1984) Identification of genes and gene products necessary for bacterial bioluminescence. *Proc Natl Acad Sci USA* **81**: 4154–4158.

Fidopiastis, P.M., Miyamoto, C.M., Jobling, M.G., Meighen, E.A., and Ruby, E.G. (2002) LitR, a new transcriptional activator in Vibrio fischeri, regulates luminescence and symbiotic light organ colonization. *Molecular Microbiology* **45**: 131–143.

Folsom, J.P., Parker, A.E., and Carlson, R.P. (2014) Physiological and proteomic analysis of Escherichia coli iron-limited chemostat growth. *J Bacteriol* **196**: 2748–2761.

Fontecave, M., Coves, J., and Pierre, J.L. (1994) Ferric reductases or flavin reductases? *Biometals* **7**: 3–8.

Forman, S., Nagiec, M.J., Abney, J., Perry, R.D., and Fetherston, J.D. (2007) Analysis of the aerobactin and ferric hydroxamate uptake systems of Yersinia pestis. *Microbiology (Reading, Engl)* **153**: 2332–2341.

Foster, K.R., and Bell, T. (2012) Competition, not cooperation, dominates interactions among culturable microbial species. *Curr Biol* **22**: 1845–1850.

Freeman, J.A., and Bassler, B.L. (1999) A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in Vibrio harveyi. *Molecular Microbiology* **31**: 665–677.

Funahashi, T., Tanabe, T., Aso, H., Nakao, H., Fujii, Y., Okamoto, K., *et al.* (2003) An iron-regulated gene required for utilization of aerobactin as an exogenous siderophore in Vibrio parahaemolyticus. *Microbiology (Reading, Engl)* **149**: 1217–1225.

Funahashi, T., Tanabe, T., Shiuchi, K., Nakao, H., and Yamamoto, S. (2009) Identification and characterization of genes required for utilization of desferri-ferrichrome and aerobactin in Vibrio parahaemolyticus. *Biol Pharm Bull* **32**: 359–365.

Genuini, M., Bidet, P., Benoist, J.-F., Schlemmer, D., Lemaitre, C., Birgy, A., and Bonacorsi, S. (2019) ShiF acts as an auxiliary factor of aerobactin secretion in meningitis Escherichia coli strain S88. *BMC Microbiol* **19**: 298–8.

Ghoul, M., and Mitri, S. (2016) The Ecology and Evolution of Microbial Competition. *Trends in Microbiology* **24**: 833–845.

Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A., and Smith, H.O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* **6**: 343–345.

Gibson, F., and Magrath, D.I. (1969) The isolation and characterization of a hydroxamic acid (aerobactin) formed by Aerobacter aerogenes 62-I. *Biochim Biophys Acta* **192**: 175–184.

Gilson, L., Kuo, A., and Dunlap, P.V. (1995) AinS and a new family of autoinducer synthesis proteins. *J Bacteriol* **177**: 6946–6951.

Graf, J., and Ruby, E.G. (2000) Novel effects of a transposon insertion in the Vibrio fischeri glnD gene: defects in iron uptake and symbiotic persistence in addition to nitrogen utilization. *Molecular Microbiology* **37**: 168–179.

Greenberg, E.P., Hastings, J.W., and Ulitzur, S. (1979) Induction of luciferase synthesis in Beneckea harveyi by other marine bacteria. *Arch Microbiol* **120**: 87–91.

Hager, P.W., Calfee, M.W., and Phibbs, P.V. (2000) The Pseudomonas aeruginosa

devB/SOL homolog, pgl, is a member of the hex regulon and encodes 6-phosphogluconolactonase. *J Bacteriol* **182**: 3934–3941.

Hardin, G. (1960) The competitive exclusion principle. *Science* **131**: 1292–1297.

Hem, J.D. (1972) Chemical Factors that Influence the Availability of Iron and Manganese in Aqueous Systems. *Geol Soc America Bull* **83**: 443–450.

Hibbing, M.E., Fuqua, C., Parsek, M.R., and Peterson, S.B. (2010) Bacterial competition: surviving and thriving in the microbial jungle. *Nat Rev Microbiol* **8**: 15–25.

Imlay, J.A., Chin, S.M., and Linn, S. (1988) Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science* **240**: 640–642.

Jaques, S., and McCarter, L.L. (2006) Three new regulators of swarming in Vibrio parahaemolyticus. *J Bacteriol* **188**: 2625–2635.

Joshi, F., Archana, G., and Desai, A. (2006) Siderophore cross-utilization amongst rhizospheric bacteria and the role of their differential affinities for Fe3+ on growth stimulation under iron-limited conditions. *Curr Microbiol* **53**: 141–147.

Khare, A., and Tavazoie, S. (2015) Multifactorial Competition and Resistance in a Two-Species Bacterial System. *PLoS Genet* **11**: e1005715.

Kim, Jisun, and Park, W. (2014) Oxidative stress response in Pseudomonas putida. *Appl Microbiol Biotechnol* **98**: 6933–6946.

Kim, Juhyun, Jeon, C.O., and Park, W. (2008) Dual regulation of zwf-1 by both 2-keto-3-deoxy-6-phosphogluconate and oxidative stress in Pseudomonas putida. *Microbiology (Reading, Engl)* **154**: 3905–3916.

Kimbrough, J.H., and Stabb, E.V. (2013) Substrate specificity and function of the pheromone receptor AinR in Vibrio fischeri ES114. *J Bacteriol* **195**: 5223–5232.

Leinweber, A., Fredrik Inglis, R., and Kümmerli, R. (2017) Cheating fosters species co-existence in well-mixed bacterial communities. *ISME J* **11**: 1179–1188.

Lenski, R.E. (2019) The *E. coli* long-term experimental evolution project site. *http://myxo.css.msu.edu/ecoli*.

Lilley, B.N., and Bassler, B.L. (2000) Regulation of quorum sensing in Vibrio harveyi by LuxO and Sigma-54. *Molecular Microbiology* **36**: 940–954.

Lin, E.C. (1976) Glycerol dissimilation and its regulation in bacteria. *Annu Rev Microbiol* **30**: 535–578.

Lin, Y.H., Miyamoto, C., and Meighen, E.A. (2000a) Purification and characterization of a luxO promoter binding protein LuxT from Vibrio harveyi. *Protein Expr Purif* **20**: 87–94.

Lin, Y.H., Miyamoto, C., and Meighen, E.A. (2000b) Cloning and functional studies of a luxO regulator LuxT from Vibrio harveyi. *Biochim Biophys Acta* **1494**: 226–235.

Logan, S.L., Thomas, J., Yan, J., Baker, R.P., Shields, D.S., Xavier, J.B., *et al.* (2018) The Vibrio cholerae type VI secretion system can modulate host intestinal mechanics to displace gut bacterial symbionts. *Proc Natl Acad Sci USA* **115**: E3779–E3787.

Lyell, N.L., Dunn, A.K., Bose, J.L., Vescovi, S.L., and Stabb, E.V. (2008) Effective mutagenesis of Vibrio fischeri by using hyperactive mini-Tn5 derivatives. *Appl Environ Microbiol* **74**: 7059–7063.

Mandel, M.J., Wollenberg, M.S., Stabb, E.V., Visick, K.L., and Ruby, E.G. (2009) A single regulatory gene is sufficient to alter bacterial host range. *Nature* **458**: 215–218.

McCarter, L.L. (1998) OpaR, a homolog of Vibrio harveyi LuxR, controls opacity of Vibrio parahaemolyticus. *J Bacteriol* **180**: 3166–3173.

McFall-Ngai, M.J., and Ruby, E.G. (1991) Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. *Science* **254**: 1491–1494.

Medema, M.H., Blin, K., Cimermancic, P., de Jager, V., Zakrzewski, P., Fischbach, M.A., *et al.* (2011) antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. *Nucleic Acids Res* **39**: W339–46.

Moon, Y.H., Tanabe, T., Funahashi, T., Shiuchi, K.I., Nakao, H., and Yamamoto, S. (2004) Identification and Characterization of Two Contiguous Operons Required for Aerobactin Transport and Biosynthesis in Vibrio mimicus. *Microbiol Immunol* **48**: 389–398.

Nadell, C.D., and Bassler, B.L. (2011) A fitness trade-off between local competition and dispersal in Vibrio cholerae biofilms. *Proc Natl Acad Sci USA* **108**: 14181–14185.

Naka, H., Actis, L.A., and Crosa, J.H. (2013) The anguibactin biosynthesis and transport genes are encoded in the chromosome of Vibrio harveyi: a possible evolutionary origin for the pJM1 plasmid–encoded system of Vibrio anguillarum? *MicrobiologyOpen* **2**: 182–194.

Neilands, J.B. (1995) Siderophores: structure and function of microbial iron transport compounds. *J Biol Chem* **270**: 26723–26726.

Niehus, R., Picot, A., Oliveira, N.M., Mitri, S., and Foster, K.R. (2017) The evolution of siderophore production as a competitive trait. *Evolution* **71**: 1443–1455.

O'Toole, G.A., Pratt, L.A., Watnick, P.I., Newman, D.K., Weaver, V.B., and Kolter, R. (1999) Genetic approaches to study of biofilms. *Methods Enzymol* **310**: 91–109.

1089 Okujo, N., and Yamamoto, S. (1994) Identification of the siderophores from Vibrio
1090 hollisae and Vibrio mimicus as aerobactin. *FEMS Microbiol Lett* **118**: 187–192.

1091 Pankey, M.S., Foxall, R.L., Ster, I.M., Perry, L.A., Schuster, B.M., Donner, R.A., *et al.*
1092 (2017) Host-selected mutations converging on a global regulator drive an adaptive leap
1093 towards symbiosis in bacteria. *Elife* **6**: 2937.

1094 Payne, S.M. (1994) Detection, isolation, and characterization of siderophores. *Methods*
1095 *Enzymol* **235**: 329–344.

1096 Payne, S.M., Mey, A.R., and Wyckoff, E.E. (2016) Vibrio Iron Transport: Evolutionary
1097 Adaptation to Life in Multiple Environments. *Microbiol Mol Biol Rev* **80**: 69–90.

1098 Pfeiffer, T., Schuster, S., and Bonhoeffer, S. (2001) Cooperation and competition in the
1099 evolution of ATP-producing pathways. *Science* **292**: 504–507.

1100 Ramesh, A., Loganathan, B.G., and Venugopalan, V.K. (1989) Seasonal distribution of
1101 luminous bacteria in the sediments of a tropical estuary. *J Gen Appl Microbiol* **35**: 363–
1102 368.

1103 Ramos, J.L., Martínez-Bueno, M., Molina-Henares, A.J., Terán, W., Watanabe, K.,
1104 Zhang, X., *et al.* (2005) The TetR Family of Transcriptional Repressors. *Microbiol Mol*
1105 *Biol Rev* **69**: 326–356.

1106 Rijkenberg, M.J.A., Middag, R., Laan, P., Gerringa, L.J.A., van Aken, H.M., Schoemann,
1107 V., *et al.* (2014) The distribution of dissolved iron in the West Atlantic Ocean. *PLoS ONE*
1108 **9**: e101323.

1109 Ruby, E.G., and Lee, K.H. (1998) The Vibrio fischeri-Euprymna scolopes Light Organ
1110 Association: Current Ecological Paradigms. *Appl Environ Microbiol* **64**: 805–812.

1111 Saha, R., Saha, N., Donofrio, R.S., and Bestervelt, L.L. (2013) Microbial siderophores: a
1112 mini review. *J Basic Microbiol* **53**: 303–317.

1113 Schiessl, K.T., Janssen, E.M.-L., Kraemer, S.M., McNeill, K., and Ackermann, M. (2017)
1114 Magnitude and Mechanism of Siderophore-Mediated Competition at Low Iron Solubility
1115 in the Pseudomonas aeruginosa Pyochelin System. *Front Microbiol* **8**: 1964.

1116 Schröder, I., Johnson, E., and de Vries, S. (2003) Microbial ferric iron reductases.
1117 *FEMS Microbiol Rev* **27**: 427–447.

1118 Schwyn, B., and Neilands, J.B. (1987) Universal chemical assay for the detection and
1119 determination of siderophores. *Anal Biochem* **160**: 47–56.

1120 Sexton, D.J., and Schuster, M. (2017) Nutrient limitation determines the fitness of
1121 cheaters in bacterial siderophore cooperation. *Nat Commun* **8**: 230–8.

1122 Silhavy, T.J., Berman, M.L., and Enquist, L.W. (1984) *Experiments with Gene Fusions.*

Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sogin, M.L., Morrison, H.G., Huber, J.A., Mark Welch, D., Huse, S.M., Neal, P.R., *et al.* (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proc Natl Acad Sci USA* **103**: 12115–12120.

Spyrou, G., Haggård-Ljungquist, E., Krook, M., Jörnvall, H., Nilsson, E., and Reichard, P. (1991) Characterization of the flavin reductase gene (fre) of Escherichia coli and construction of a plasmid for overproduction of the enzyme. *J Bacteriol* **173**: 3673–3679.

Stabb, E.V., and Ruby, E.G. (2002) RP4-based plasmids for conjugation between Escherichia coli and members of the Vibrionaceae. *Methods Enzymol* **358**: 413–426.

Stocker, R., and Seymour, J.R. (2012) Ecology and physics of bacterial chemotaxis in the ocean. *Microbiol Mol Biol Rev* **76**: 792–812.

Stubbendieck, R.M., and Straight, P.D. (2016) Multifaceted Interfaces of Bacterial Competition. *J Bacteriol* **198**: 2145–2155.

Sunagawa, S., Coelho, L.P., Chaffron, S., Kultima, J.R., Labadie, K., Salazar, G., *et al.* (2015) Ocean plankton. Structure and function of the global ocean microbiome. *Science* **348**: 1261359–1261359.

Suzuki, K., Tanabe, T., Moon, Y.H., Funahashi, T., Nakao, H., Narimatsu, S., and Yamamoto, S. (2006) Identification and transcriptional organization of aerobactin transport and biosynthesis cluster genes of Vibrio hollisae. *Res Microbiol* **157**: 730–740.

Tanabe, T., Funahashi, T., Miyamoto, K., Tsujibo, H., and Yamamoto, S. (2011) Identification of genes, desR and desA, required for utilization of desferrioxamine B as a xenosiderophore in Vibrio furnissii. *Biol Pharm Bull* **34**: 570–574.

Tanabe, T., Kato, A., Shiuchi, K., Miyamoto, K., Tsujibo, H., Maki, J., *et al.* (2014) Regulation of the expression of the Vibrio parahaemolyticus peuA gene encoding an alternative ferric enterobactin receptor. *PLoS ONE* **9**: e105749.

Tanabe, T., Naka, A., Aso, H., Nakao, H., Narimatsu, S., Inoue, Y., *et al.* (2005) A novel aerobactin utilization cluster in Vibrio vulnificus with a gene involved in the transcription regulation of the iutA homologue. *Microbiol Immunol* **49**: 823–834.

Thode, S.K., Rojek, E., Kozlowski, M., Ahmad, R., and Haugen, P. (2018) Distribution of siderophore gene systems on a Vibrionaceae phylogeny: Database searches, phylogenetic analyses and evolutionary perspectives. *PLoS ONE* **13**: e0191860.

Thompson, F.L., Iida, T., and Swings, J. (2004) Biodiversity of Vibrios. *Microbiol Mol Biol Rev* **68**: 403–431.

Travisano, M., and Lenski, R.E. (1996) Long-term experimental evolution in Escherichia coli. IV. Targets of selection and the specificity of adaptation. *Genetics* **143**: 15–26.

Traxler, M.F., Seyedsayamdost, M.R., Clardy, J., and Kolter, R. (2012) Interspecies modulation of bacterial development through iron competition and siderophore piracy. *Molecular Microbiology* **86**: 628–644.

Tu, K.C., and Bassler, B.L. (2007) Multiple small RNAs act additively to integrate sensory information and control quorum sensing in Vibrio harveyi. *Genes Dev* **21**: 221–233.

Veldkamp, H., and Jannasch, H.W. (1972) Mixed culture studies with the chemostat. *J Appl Chem* **22**: 105–123.

Weaver, V.B., and Kolter, R. (2004) Burkholderia spp. alter Pseudomonas aeruginosa physiology through iron sequestration. *J Bacteriol* **186**: 2376–2384.

Westrich, J.R., Ebling, A.M., Landing, W.M., Joyner, J.L., Kemp, K.M., Griffin, D.W., and Lipp, E.K. (2016) Saharan dust nutrients promote Vibrio bloom formation in marine surface waters. *Proc Natl Acad Sci USA* **113**: 5964–5969.

Whitman, W.B., Coleman, D.C., and Wiebe, W.J. (1998) Prokaryotes: the unseen majority. *Proc Natl Acad Sci USA* **95**: 6578–6583.

Wier, A.M., Nyholm, S.V., Mandel, M.J., Massengo-Tiassé, R.P., Schaefer, A.L., Koroleva, I., *et al.* (2010) Transcriptional patterns in both host and bacterium underlie a daily rhythm of anatomical and metabolic change in a beneficial symbiosis. *Proc Natl Acad Sci USA* **107**: 2259–2264.

Wooldridge, K.G., Morrissey, J.A., and Williams, P.H. (1992) Transport of ferric-aerobactin into the periplasm and cytoplasm of Escherichia coli K12: role of envelope-associated proteins and effect of endogenous siderophores. *J Gen Microbiol* **138**: 597–603.

Zane, H.K., Naka, H., Rosconi, F., Sandy, M., Haygood, M.G., and Butler, A. (2014) Biosynthesis of amphi-enterobactin siderophores by Vibrio harveyi BAA-1116: identification of a bifunctional nonribosomal peptide synthetase condensation domain. *J Am Chem Soc* **136**: 5615–5618.

Figure Legends

Fig. 1. *V. fischeri* ES114 culture fluids prevent growth of vibrio species in minimal marine medium.

A. Representative *V. harveyi* BB120 cultures (left) and the corresponding optical density (OD_{600}) readings (right) following growth for 16 h in minimal marine medium supplemented with 10% (v/v) of the indicated culture fluid. ES114 and MJ11 denote *V. fischeri* ES114 and *V. fischeri* MJ11, respectively. For the no addition control, 10% (v/v) sterile minimal marine medium was supplied instead of culture fluids.

B. Growth of the indicated bacterial species after 16 h in minimal marine medium supplied with 10% (v/v) sterile minimal marine medium (black) or 10% (v/v) culture fluids from *V. fischeri* ES114 grown in minimal marine medium (gray). In A and B, error bars represent standard deviations for three biological replicates. Unpaired two-tailed *t* tests were performed comparing the treated conditions to the no addition controls. P-values: ns ≥ 0.05, **** < 0.0001.

Fig. 2. *V. fischeri* ES114 produces a siderophore that inhibits *V. harveyi* growth.

A. Schematic of the *V. fischeri* ES114 aerobactin biosynthetic operon. The arrows depict the locations of five independent transposon insertions identified in the screen.

B. *V. harveyi* growth in minimal marine medium supplemented with 10% (v/v) culture fluids from WT *V. fischeri* ES114 or the indicated *V. fischeri* ES114 mutant strain. In the case of the no addition control, 10% (v/v) minimal marine medium was added.

C. CAS assay quantitation of siderophore present in culture fluids collected from WT *V. fischeri* ES114, the indicated mutant *V. fischeri* ES114 strains, or *V. fischeri* MJ11.

D. *V. harveyi* growth in minimal marine medium supplemented with 10% (v/v) culture fluids prepared from WT *V. fischeri* ES114. The indicated concentrations of ferrous (Fe^{2+}, no siderophore required) sulfate were added simultaneously to the cell-free culture fluids. In the case of the no addition control, 10% (v/v) minimal marine medium was added. In B, C, and D, error bars represent standard deviations for three biological replicates. Unpaired two-tailed *t* tests were performed comparing the treated conditions to the no addition control (B), mutants to WT (C), and iron-treated samples to the

untreated (0 μM) control sample (D). P values: ns ≥ 0.05, * < 0.05, *** < 0.001, **** < 0.0001.

Fig. 3. Fur represses *V. fischeri* ES114 siderophore production under iron-replete conditions.

A. CAS assay quantitation of siderophore present in culture fluids prepared from WT (black) or Δ*fur* (gray) *V. fischeri* ES114 grown in minimal marine medium supplemented with the indicated concentrations of ferrous (Fe^{2+}, no siderophore required) sulfate. To account for increased growth in response to increasing iron concentrations, values were normalized to a siderophore unit value of 1 for the Δ*fur* mutant at each iron concentration.

B. Relative fluorescence values (mVenus/OD_{600}) for WT (black) or Δ*fur* (gray) *V. fischeri* ES114 strains carrying an *iucA*'-*mVenus* transcriptional reporter on a plasmid. The strains were grown in minimal marine medium supplemented with the indicated concentrations of ferrous (Fe^{2+}, no siderophore required) sulfate. Values represent the relative fluorescence at OD_{600} = 0.4 for each condition. For A and B, error bars represent standard deviations for three biological replicates. Unpaired two-tailed *t* tests were performed comparing WT to Δ*fur* for each treatment. P values: ns ≥ 0.05, ** < 0.01, **** < 0.0001.

Fig. 4. LuxT, YebK, Fre, and GlpK are activators of siderophore production in *V. fischeri* ES114.

A. CAS assay quantitation of siderophore levels in culture fluids collected from the indicated *V. fischeri* ES114 strains.

B. *V. harveyi* growth after 16 h in minimal marine medium supplemented with 10% (v/v) culture fluids prepared from the indicated *V. fischeri* ES114 strains. In the case of the no addition control, 10% (v/v) sterile minimal marine medium was added.

C. Relative fluorescence values (mVenus/OD_{600}) of the indicated *V. fischeri* ES114 strains carrying an *iucA*'-*mVenus* transcriptional reporter on a plasmid. Values represent relative fluorescence when OD_{600} = 0.4 for each sample.

D. As in C, except that the strains harbor a translational *iucA'-'mVenus* reporter. In all

1248 panels, error bars represent standard deviations of three biological replicates. Unpaired
1249 two-tailed *t* tests were performed comparing mutants to WT (A, B, C, and D). P values:
1250 ns ≥ 0.05, *** < 0.001, **** < 0.0001.
1251
1252 **Fig. 5.** IutA and FhuCDB are sufficient for aerobactin cheating.
1253 A. The *V. fischeri* ES114 aerobactin biosynthetic gene cluster, depicted to scale.
1254 Homologs identified in *V. parahaemolyticus* BB22OP and *V. vulnificus* YJ016 are shown
1255 along with the percent positive similarity in amino acid sequence to the corresponding
1256 *V. fischeri* ES114 homolog.
1257 B. Growth after 16 h of the indicated *V. parahaemolyticus* BB22OP and *V. fischeri*
1258 ES114 strains in minimal marine medium supplied with 10% (v/v) minimal marine
1259 medium (black) or culture fluids from WT *V. fischeri* ES114 (gray). The pControl plasmid
1260 is the empty parent vector. p*iutA* carries the *V. fischeri* ES114 *iutA* gene expressed from
1261 its native promoter.
1262 C. Growth after 16 h of *V. harveyi* harboring the indicated plasmids in minimal marine
1263 medium supplemented with 10% (v/v) minimal marine medium (black) or *V. fischeri*
1264 ES114 culture fluids (gray). The pControl plasmid is the empty parent vector. p*iutA,*
1265 p*fhuCDB,* and p*fhuCDBiutA* carry the indicated genes expressed from their native
1266 promoters. In B and C, error bars represent standard deviations for three biological
1267 replicates. Unpaired two-tailed *t* tests were performed comparing the no addition control
1268 samples to the treated samples for each condition. P values: ns ≥ 0.05, **** < 0.0001.
1269
1270 **Fig. 6.** *aerE* encodes an MFS transporter that exports aerobactin.
1271 A. CAS assay quantitation of siderophore in culture fluids collected from the indicated *V.*
1272 *fischeri* ES114 strains grown in minimal marine medium.
1273 B. CAS assay quantitation of intracellular siderophore levels in the strains from A. In A
1274 and B, error bars represent standard deviations for three biological replicates. Unpaired
1275 two-tailed *t* tests were performed comparing mutants to WT. P values: ns ≥ 0.05, *** <
1276 0.001, **** < 0.0001.
1277
1278 **Fig. 7.** Differences in aerobactin production between *V. fischeri* ES114 and *V. fischeri*

MJ11 stem from differences at the protein level.

A. CAS assay quantitation of siderophore levels in culture fluids from WT (black bar) or Δ*iucABCD* (gray bars) *V. fischeri* ES114 harboring the indicated plasmids. The pControl plasmid is the empty parent vector. The p*iucABCD*(ES114) and p*iucABCD*(MJ11) plasmids carry *iucABCD* from the indicated strain under the IPTG inducible *tac* promoter. Cultures were grown in minimal marine medium with 0.5 mM IPTG to induce expression.

B. CAS assay quantitation of siderophore levels in *V. fischeri* MJ11 culture fluids. Plasmids and growth conditions as in A. In A and B, error bars represent standard deviations for three biological replicates. Unpaired two-tailed *t* tests were performed comparing the strains harboring p*iucABCD*(ES114) and p*iucABCD*(MJ11) to the pControl strain. P values: * < 0.05, *** < 0.001, **** < 0.0001.

Fig. 8. *V. fischeri* ES114 aerobactin production confers a competitive advantage against *V. harveyi* when iron is limiting.

Competition experiments between *V. harveyi* and either WT (black circles) or Δ*iucABCD* (white circles) *V. fischeri* ES114 under the indicated growth conditions. For each competition, the selection rate constant (24 h^{-1}) is plotted for the *V. fischeri* ES114 strain compared to *V. harveyi*. Error bars represent standard deviations of five biological replicates. Unpaired two-tailed *t* tests were performed comparing the *V. harveyi/V. fischeri* ES114 WT competition to the *V. harveyi/V. fischeri* ES114 Δ*iucABCD* competition for each growth condition. P values: ns ≥ 0.05, **** < 0.0001.

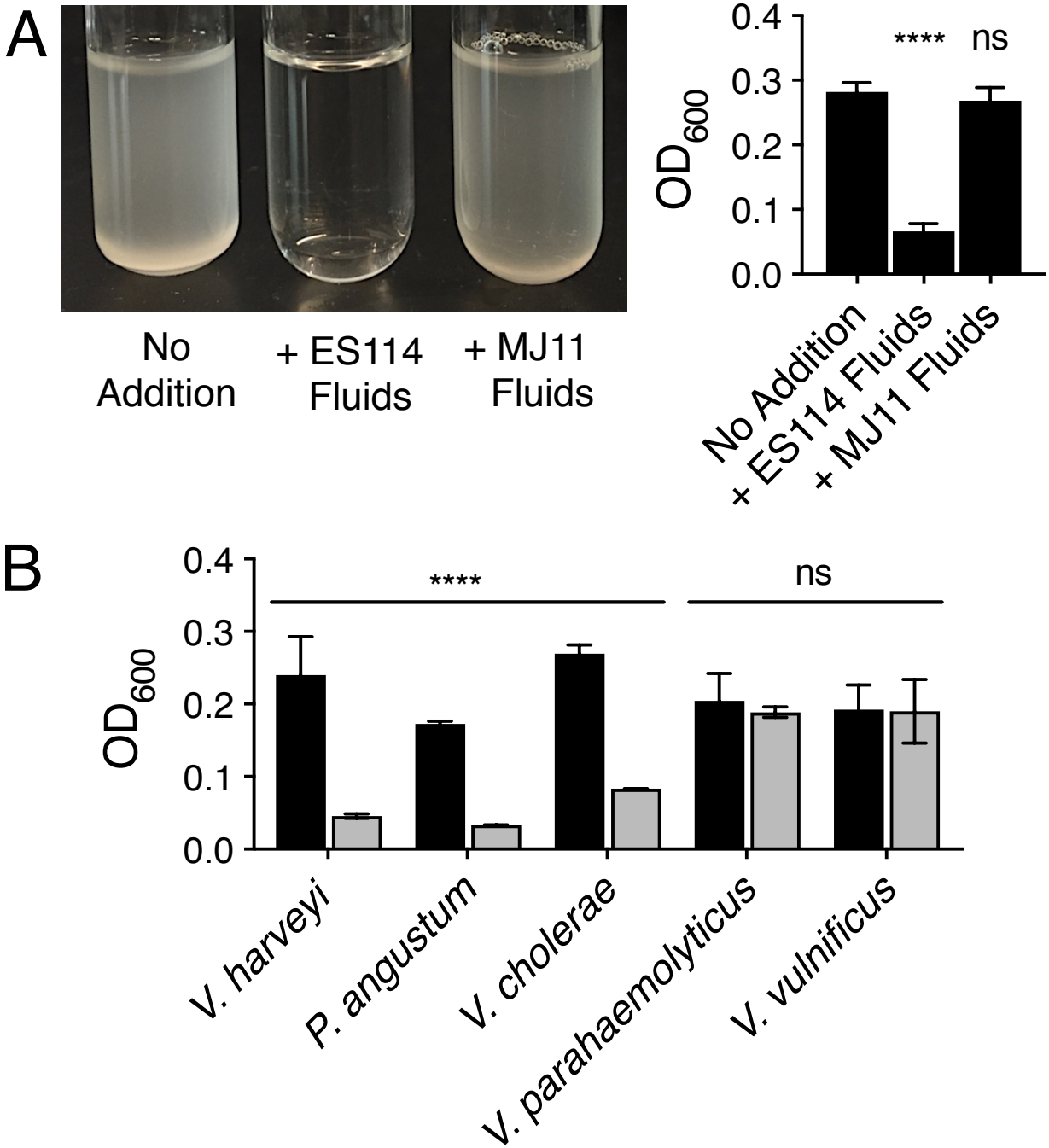

A
No Addition
+ ES114 Fluids
+ MJ11 Fluids
OD600
0.4
0.3
0.2
0.1
0.0
****
ns
No Addition
+ ES114 Fluids
+ MJ11 Fluids
B
OD600
0.4
0.3
0.2
0.1
0.0
****
ns
V. harveyi
P. angustum
V. cholerae
V. parahaemolyticus
V. vulnificus

Figure 1

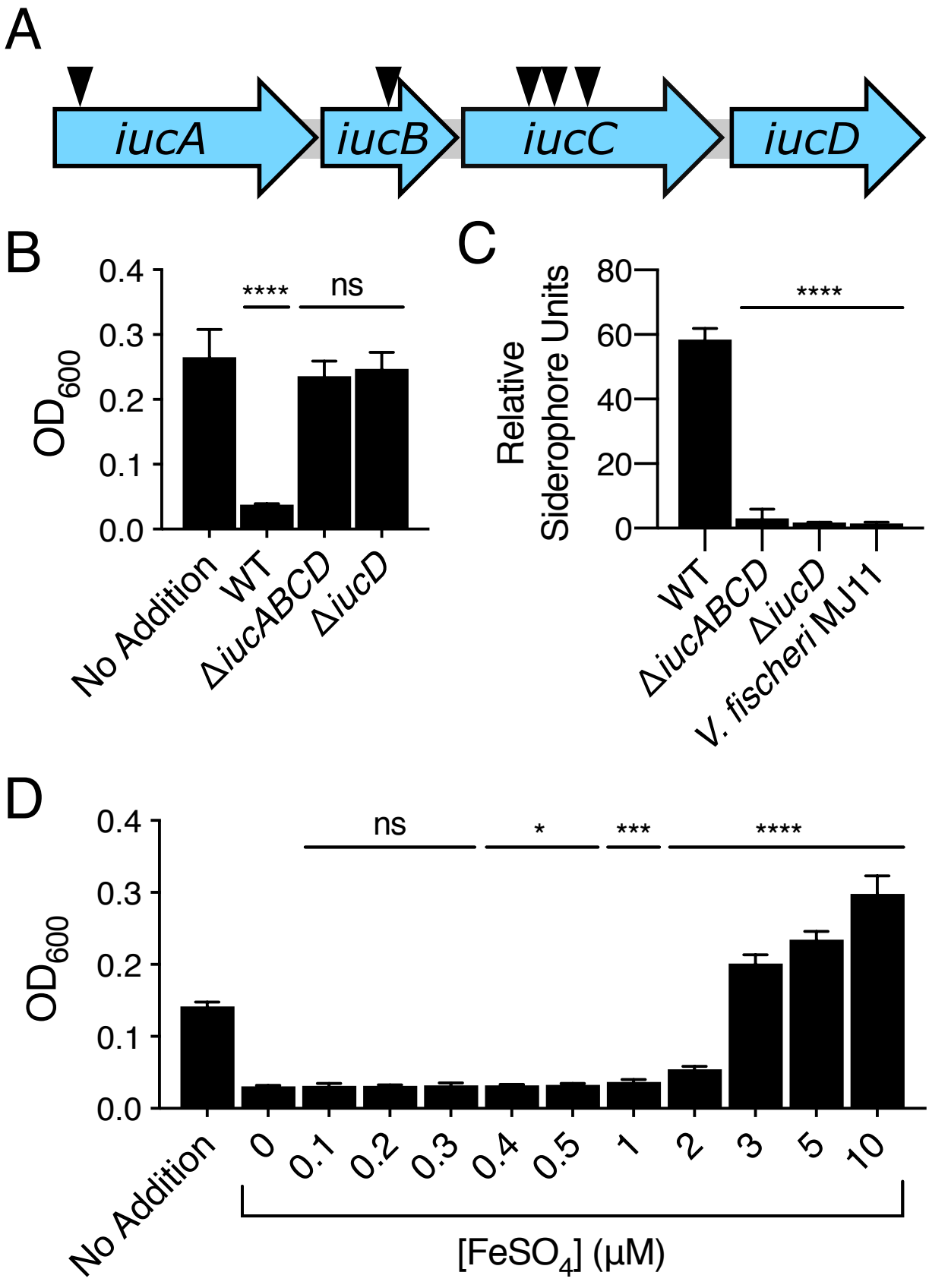

A
iucA
iucB
iucC
iucD
B
OD600
0.4
0.3
0.2
0.1
0.0
****
ns
No Addition
WT
ΔiucABCD
ΔiucD
C
Relative Siderophore Units
80
60
40
20
0
****
WT
ΔiucABCD
ΔiucD
V. fischeri MJ11
D
OD600
0.4
0.3
0.2
0.1
0.0
ns
*
***
****
No Addition
0
0.1
0.2
0.3
0.4
0.5
1
2
3
5
10
[FeSO4] (μM)

Figure 2

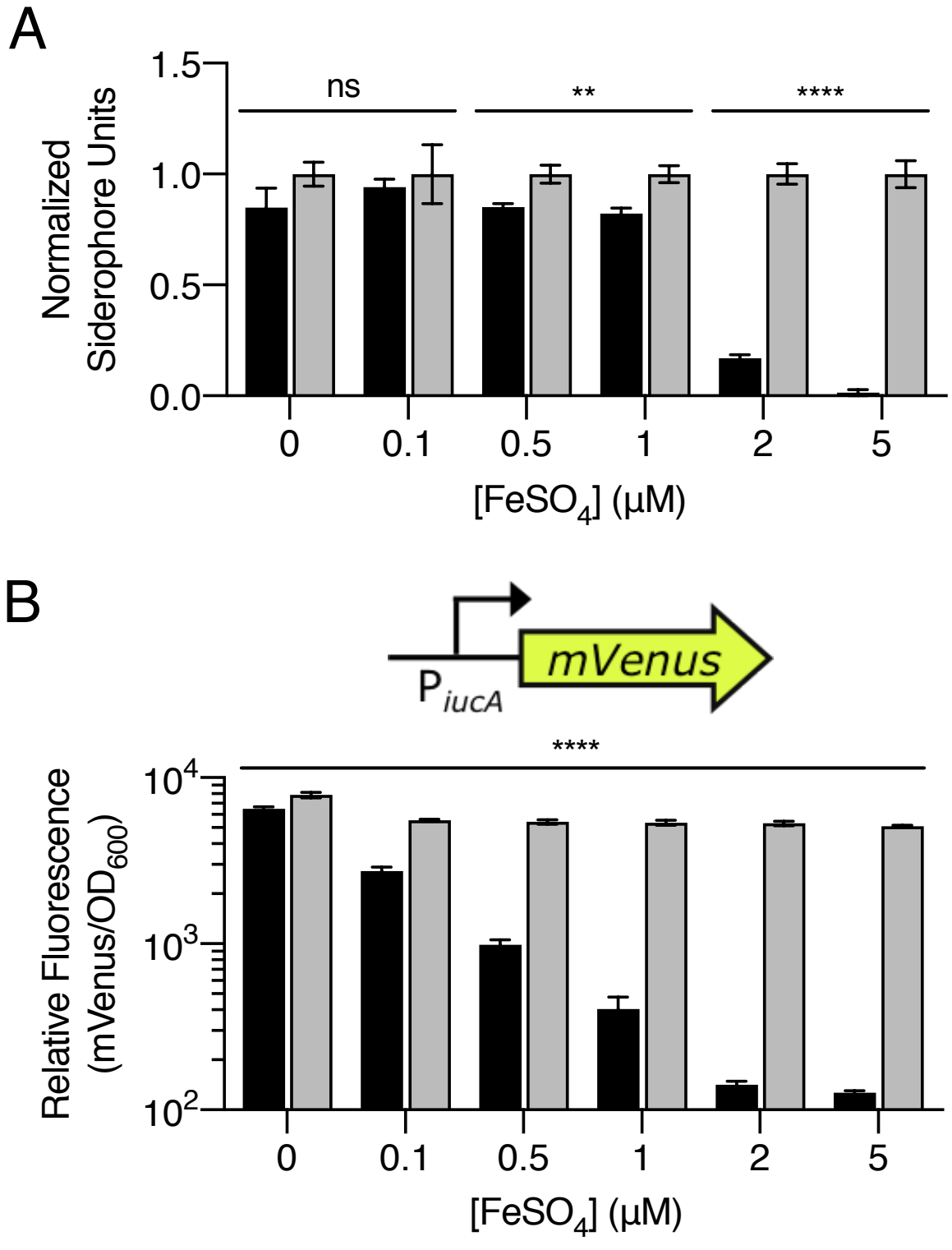

A
Normalized Siderophore Units
1.5
1.0
0.5
0.0
ns
**
****
0
0.1
0.5
1
2
5
[FeSO4] (µM)
B
mVenus
PiucA
****
Relative Fluorescence (mVenus/OD600)
10^4
10^3
10^2
0
0.1
0.5
1
2
5
[FeSO4] (µM)

Figure 3

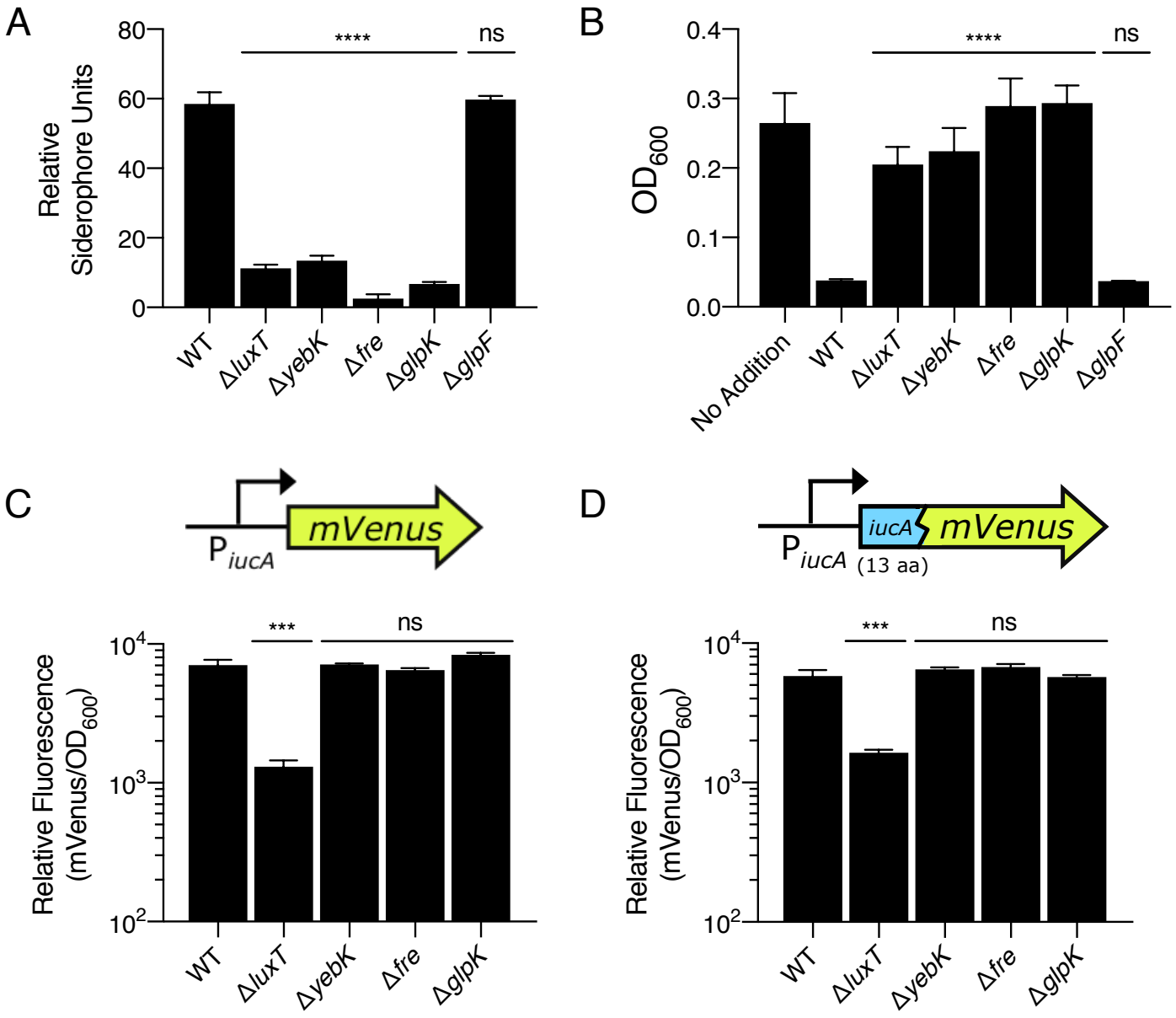

A
Relative Siderophore Units
80
60
40
20
0
****
ns
WT
ΔluxT
ΔyebK
Δfre
ΔglpK
ΔglpF
B
OD600
0.4
0.3
0.2
0.1
0.0
****
ns
No Addition
WT
ΔluxT
ΔyebK
Δfre
ΔglpK
ΔglpF
C
PiucA
mVenus
Relative Fluorescence (mVenus/OD600)
10^4
10^3
10^2
***
ns
WT
ΔluxT
ΔyebK
Δfre
ΔglpK
D
PiucA (13 aa)
iucA
mVenus
Relative Fluorescence (mVenus/OD600)
10^4
10^3
10^2
***
ns
WT
ΔluxT
ΔyebK
Δfre
ΔglpK

Figure 4

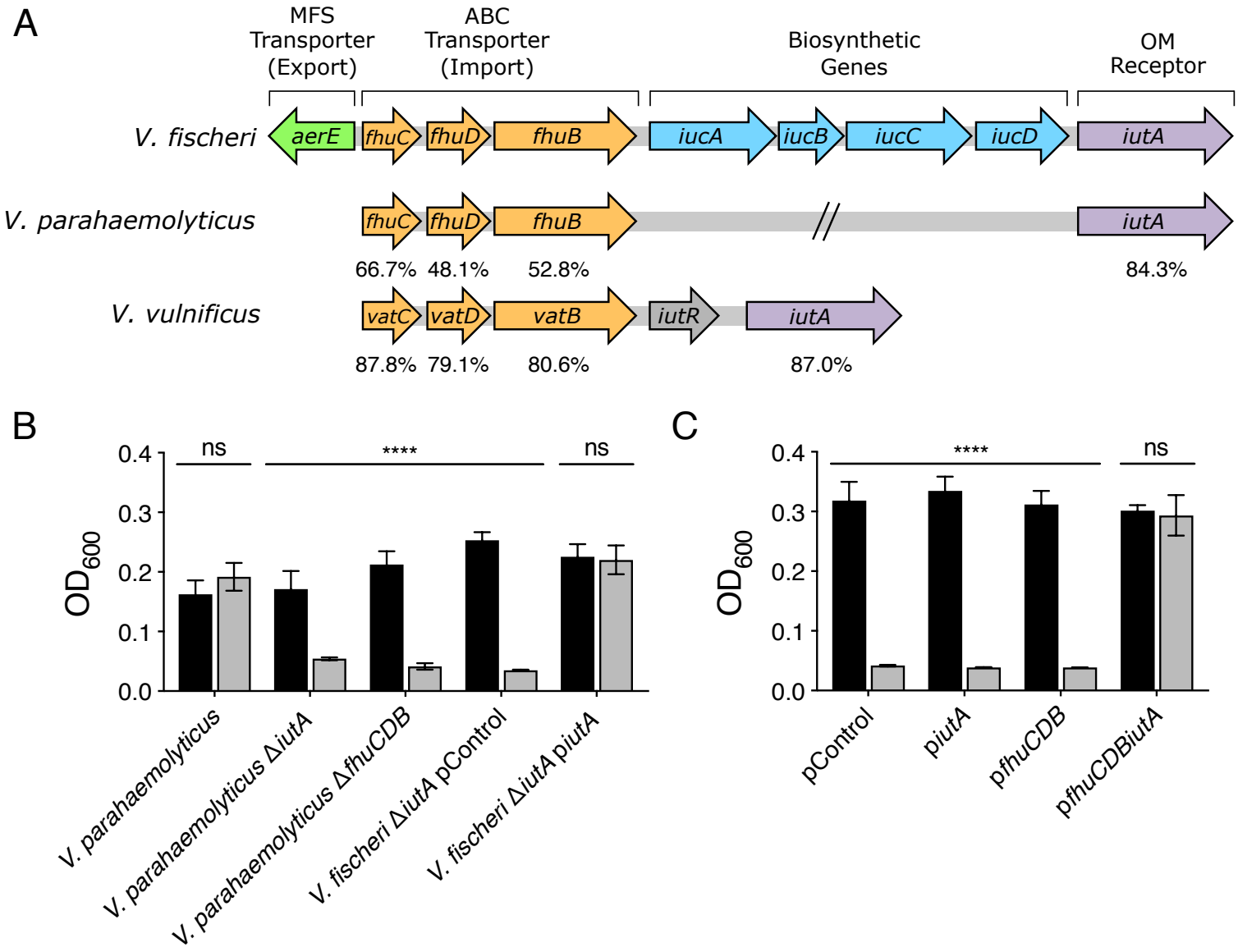

A
MFS Transporter (Export)
ABC Transporter (Import)
Biosynthetic Genes
OM Receptor
V. fischeri
aerE
fhuC
fhuD
fhuB
iucA
iucB
iucC
iucD
iutA
V. parahaemolyticus
fhuC
fhuD
fhuB
iutA
66.7%
48.1%
52.8%
84.3%
V. vulnificus
vatC
vatD
vatB
iutR
iutA
87.8%
79.1%
80.6%
87.0%
B
ns
****
ns
OD600
0.4
0.3
0.2
0.1
0.0
V. parahaemolyticus
V. parahaemolyticus ΔiutA
V. parahaemolyticus ΔfhuCDB
V. fischeri ΔiutA pControl
V. fischeri ΔiutA piutA
C
****
ns
OD600
0.4
0.3
0.2
0.1
0.0
pControl
piutA
pfhuCDB
pfhuCDBiutA

Figure 5

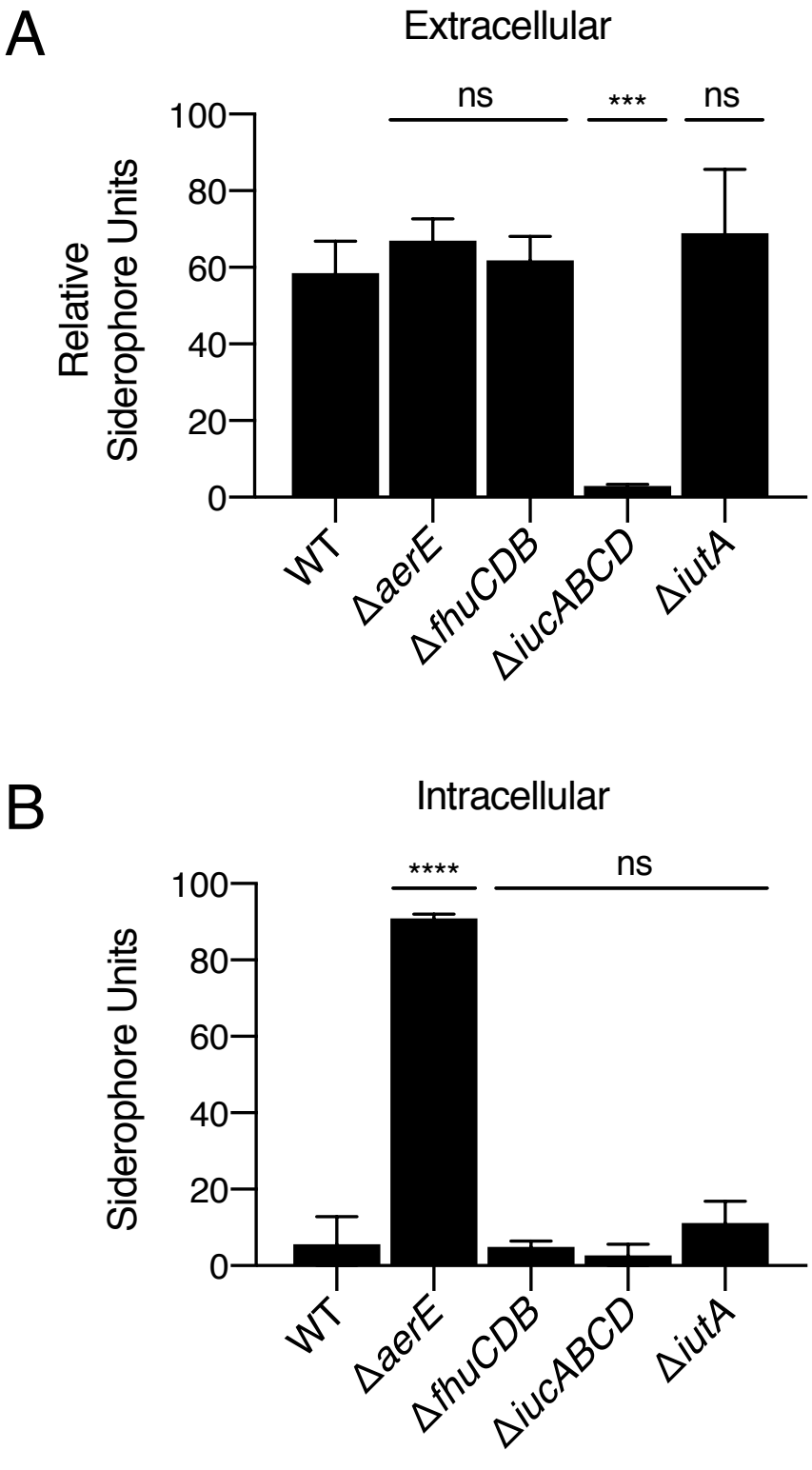
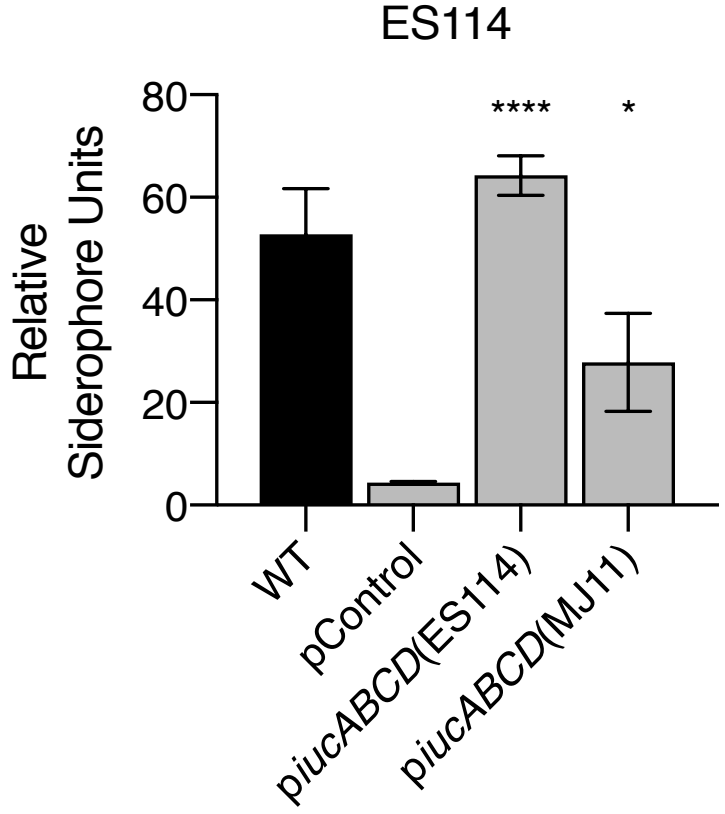

A
Extracellular
ns
***
ns
Relative Siderophore Units
100
80
60
40
20
0
WT
ΔaerE
ΔfhuCDB
ΔiucABCD
ΔiutA
B
Intracellular
****
ns
Siderophore Units
100
80
60
40
20
0
WT
ΔaerE
ΔfhuCDB
ΔiucABCD
ΔiutA

Figure 6

A


ES114
Relative Siderophore Units
80
60
40
20
0
****
*
WT
pControl
piucABCD(ES114)
piucABCD(MJ11)

B

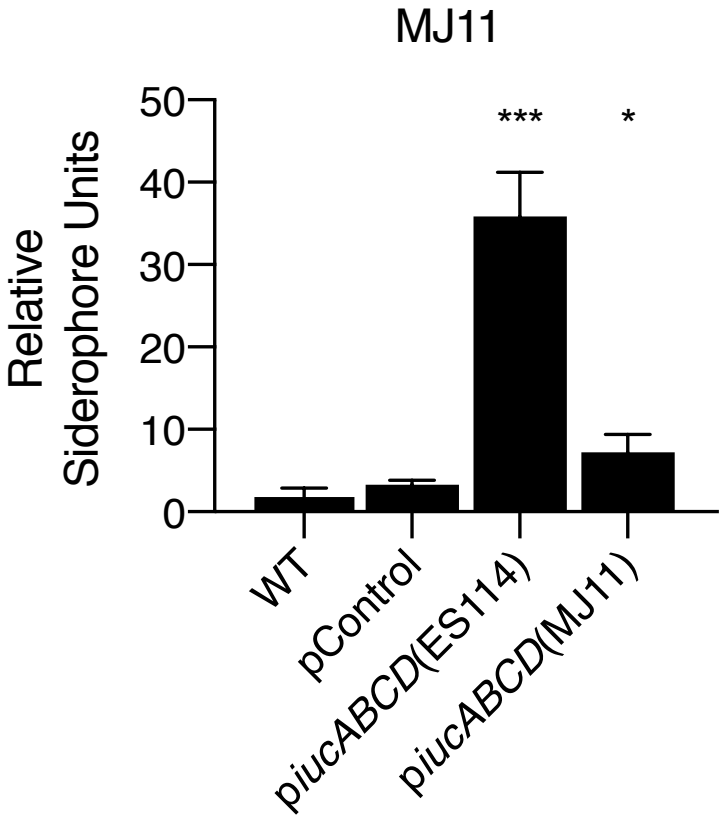

MJ11
Relative Siderophore Units
50
40
30
20
10
0
***
*
WT
pControl
piucABCD(ES114)
piucABCD(MJ11)

Figure 7

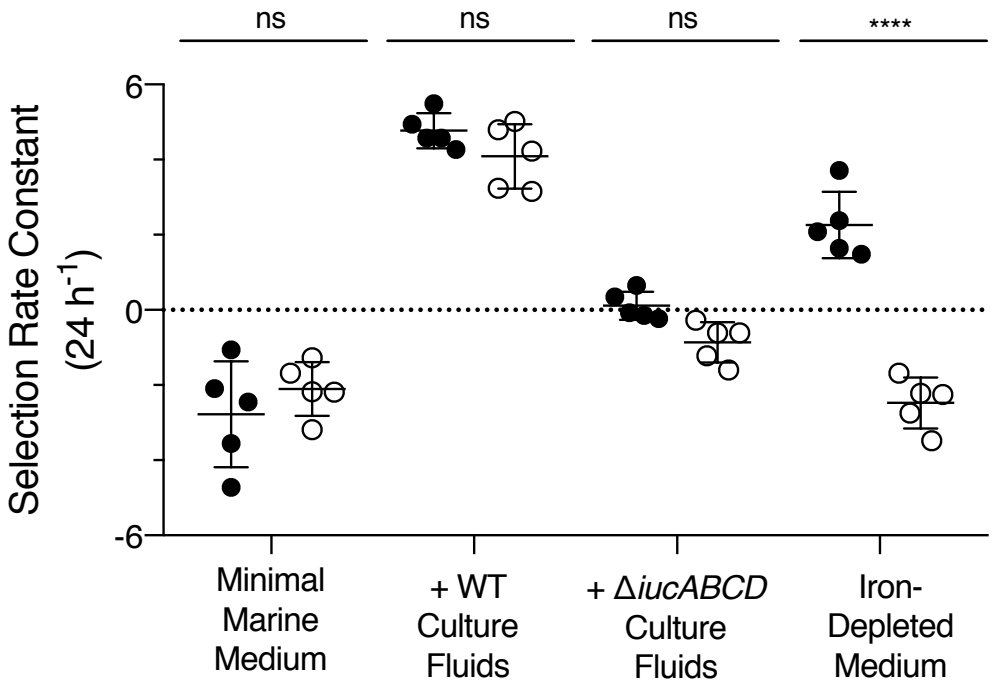

ns
ns
ns
****
6
0
-6
Selection Rate Constant
(24 h-1)
Minimal Marine Medium
+ WT Culture Fluids
+ ΔiucABCD Culture Fluids
Iron-Depleted Medium

Figure 8