Quorum sensing and iron regulate a two-for-one siderophore gene cluster in *Vibrio harveyi*

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The secretion of small Fe-binding molecules called siderophores is an important microbial strategy for survival in Fe-limited environments. Siderophore production is often regulated by quorum sensing (QS), a microbial counting technique that allows organisms to alter gene expression based on cell density. However, the identity and quantities of siderophores produced under QS regulation are rarely studied in the context of their roles in Fe uptake. We investigated the link between QS, siderophores, and Fe uptake in the model marine organism *Vibrio harveyi* where QS is thought to repress siderophore production. We find that *V. harveyi* uses a single QS- and Fe-repressed gene cluster to produce both cell-associated siderophores (amphiphilic enterobactins) as well as several related soluble siderophores, which we identify and quantify using liquid chromatography-coupled (LC)-MS as well as tandem high-resolution MS (LC-HR-MS/MS). Measurements of siderophore production show that soluble siderophores are present at ~100× higher concentrations than amphi-enterobactin and that over the course of growth *V. harveyi* decreases amphi-enterobactin concentrations but accumulates soluble siderophores. Fe radio-tracer uptake experiments demonstrate that these soluble siderophores play a significant role in Fe uptake and that the QS-dictated concentrations of soluble siderophores in stationary phase are near the limit of cellular uptake capacities. We propose that cell-associated and soluble siderophores are beneficial to *V. harveyi* in different environmental and growth contexts and that QS allows *V. harveyi* to exploit “knowledge” of its population size to avoid unnecessary siderophore production.

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The potential benefit of QS regulation of siderophores is widely appreciated (for example, refs. 16–19). However, we still do not fully understand why QS represses siderophore production in some organisms but stimulates it in others. In fact, arguments have been made in favor of both scenarios. Stimulation of production at high cell density might allow bacteria to ensure that siderophores are used by members of their own species (18). In contrast, repression of siderophore production at high cell density could allow bacteria to divert resources toward growth or dispersal (16). The identity and concentrations of siderophores produced under QS regulation are germane to understanding their utility in these contexts but have only been explored in a few species. Potential coregulation of siderophore production by Fe, while expected, has also been underexplored. Here, we investigate the types and quantities of siderophores produced in response to QS and Fe as well as their efficacy for Fe uptake in the model marine bacterium *Vibrio harveyi* BAA-1116. QS in *V. harveyi* is well studied and is known to regulate numerous cellular processes, including the production of siderophores (14). Using the chrome azuro S (CAS) assay (20), a proxy for the presence of Fe-binding molecules, Lilley and Bassler (14) showed that the accumulation of QS molecules leads to a decrease in siderophore production. More recently, Zane et al. (21) demonstrated that *V. harveyi* produces a suite of amphiphilic enterobactin-like siderophores (amphi-enterobactins) that are associated with the cell pellet. Work by Naka et al. (22) has shown that two siderophores | quorum sensing | iron | Vibrio harveyi | high-resolution mass spectrometry

Iron (Fe) is an essential nutrient that can limit microbial growth. Despite the abundance of Fe on the planet, the low solubility of ferric iron at circumneutral pH often leads to low bioavailability. One solution to this problem is the production of siderophores, small iron-binding molecules that solubilize Fe(III), making it bioavailable to the producing bacterium (1). Although siderophores are most commonly considered Fe-scavenging molecules, it is clear that they can also mediate microbial interactions. Many microbes take up siderophores that they do not synthesize themselves (2–5), and siderophores can be used to sequester iron from microbial competitors (5) as well as animal hosts (6). Siderophores have also been used to investigate cooperative behavior and symbiotic interactions (7–9).

The study of siderophore regulation provides a fruitful approach for understanding the multiple ways in which microbes use these molecules. One particularly intriguing finding is that in many organisms siderophore production is regulated by quorum sensing (QS), a counting technique that allows microbes to tailor gene expression to cell density (10). During the process of QS, microbes both produce and detect extracellular signaling molecules, the concentration of which is an indicator of population size. The accumulation of QS molecules often represses siderophore synthesis, leading to a decrease in their rate of production at high cell density (11–14). Conversely, increases in siderophores in response to QS have been documented in *Pseudomonas aeruginosa* (15).

**Significance**

Siderophores are small molecules used by microbes to acquire the essential metal Fe in environments ranging from infections to the open ocean. Although many bacteria use quorum sensing (QS) to regulate siderophore production, the resulting siderophore concentrations and their utility for Fe uptake have been underexplored. We report that *Vibrio harveyi* uses a “two-for-one,” QS- and Fe-repressed gene cluster to produce cell-associated siderophores at low cell density and accumulate soluble siderophores at high cell density. Fe-uptake experiments show that these soluble siderophores are used in Fe acquisition. We also find that QS repression helps prevent the accumulation of siderophore concentrations in excess of cellular uptake capacities, an explanation that may be generalizable to other bacteria.
Siderophore Biosynthetic Genes Are Repressed by Fe and QS. *V. harveyi* produces three autoinducers: 3-hydroxybutanoyl homoserine lactone (AI-1), (S)-3-hydroxytridecan-4-one (CAI-1), and (2S,4S)-2-methyl-2,3,3,4-tetrahydroxy-tetrahydrofuran borate (AI-2) (23–25). These autoinducers are integrated additively by the same regulatory circuit (26). Using a QS mutant [ΔluxM ΔluxPQ ΔcqsS (26)] that responds only to the homoserine lactone autoinducer (hereafter referred to as the QS mutant) we tested the effect of Fe and QS signal concentrations on the expression of anguibactin and amphi-enterobactin gene clusters. Fe limitation was achieved using a chemically defined medium in which background Fe was both low and chelated using EDTA (EDTA = 100 mM, FeT = 100 nM, unchelated Fe, Fe′ = 0.12 nM). Cells were cultured with: (i) no addition, (ii) Fe, (iii) HSL, or (iv) Fe and HSL. Fe and HSL were added at two concentrations: 100 and 500 nM. As expected, the addition of HSL did not have an effect on growth. However, Fe limitation clearly affects growth as cultures with 500 nM Fe grew faster than those with 0 or 100 nM Fe (Fig. 1A).

Both amphi-enterobactins and anguibactin are synthesized by nonribosomal peptide-synthetase (NRPS) enzymes. Using reverse transcription-quantitative PCR (RT-qPCR), we explored the expression of the anguibactin biosynthetic genes *aebB* and *aebF*. *AebB* encodes a bifunctional isochorismatase and aryl carrier protein with homology to the enterobactin gene *entB* (21, 27). *AebF* has homology to the enterobactin gene *entF* and encodes a four-domain NRPS (21, 28). In addition, we tested the expression of the anguibactin genes *angB* (a homolog of *aebB* and *entB*) that is used in the assembly of the anguibactin catechol moiety (29) and *angM*, which encodes peptidyl-carrier protein and condensation domain involved in the incorporation of N-hydroxymatine into anguibactin (29). The expression of both amphi-enterobactin genes was clearly down-regulated by 100 and 500 nM Fe (Fig. 1B). A similar result was observed with the addition of either 100 or 500 nM HSL. In contrast, *angB* and *angM* (anguibactin associated genes) did not respond to either Fe or HSL in the medium (Fig. 1C). Expression of anguibactin genes, while detected, was low (threshold cycle (*C_T*) values of ~30). These results indicate that the production of amphi-enterobactins is repressed by both QS and Fe, while anguibactin synthesis responds to neither.

Detection and Quantification of Siderophores. We next examined the production of amphi-enterobactins and anguibactin by LC-MS and LC-HR-MS/MS. In agreement with previous studies (21), we found several amphi-enterobactins with slight variations in the fatty-acid tail in cell pellet extracts (SI Appendix, Figs. S1 and S2). In addition to variants 1 and 4 reported by Zane et al. (21) (Fig. 2, see SI Appendix, Fig. S1 for a full suite of amphi-enterobactins), two amphi-enterobactins were also identified, which we suggest contain decanoyl (2, *m/z* = 911.3193) and tetradecanol (3, *m/z* = 967.3193) groups (Fig. 2 and SI Appendix, Fig. S1). Consistent with our RT-qPCR results, LC-MS analysis showed strong repression of amphi-enterobactins (we tracked a representative variant, *m/z* = 965.381, *SI Appendix*, Fig. S1, hereafter amphi-enterobactin) by Fe and more modest repression by HSL (Fig. 1D). Notably, our experiments use HSL as the sole QS molecule. In wild-type cultures where all three QS molecules are produced, this response is likely stronger.

We were unable to detect anguibactin (5) in *V. harveyi* supernatants prepared under several conditions, although it was readily detected in supernatant extracts from the known anguibactin producer *Vibrio anguillarum* 775. The absence of both an anguibactin gene expression response and anguibactin production implies that this gene cluster is not active under our experimental conditions (it remains unknown whether this gene cluster is active in other contexts). In contrast, the *V. harveyi aeb* gene cluster is expressed and the biosynthesis of amphi-enterobactin clearly responds to exogenous Fe and QS molecules.
Materials and Methods

Release of several inter supernatants were has been μ Structures of amphi-enterobactins, anguibactin, DHBA, and DHB-ser are proposed in this study based on MS/MS data (0.005, one-way ANOVA). There was no and or [M-2H V. harveyi to utilize the added soluble siderophores and syn-

To gain further insight into the production and use of Small Soluble Siderophores Aid in Fe Uptake. To test the utility of DHBA and DHB-Ser in Fe acquisition, we compared Fe-uptake rates in the presence and absence of 1 μM synthetic DHBA, or spent medium containing V.-harveyi–produced DHBA, in both WT cultures and cultures deficient in the production of enterobactins (ΔaebF, S1 Appendix, Fig. S1 and Table S1). In doing so, we identified several related structures (Fig. 3 and SI Appendix, Fig. S4 and Table S1), many corresponding to linearized amphi-enterobactin fragments. Collectively, our data imply that, in addition to producing amphi-enterobactins, V. harveyi secretes significantly larger quantities of DHB-Ser and DHB-Ser, as well as biosynthetic intermediates, a process that can be attributed to both premature release and degradation of the full-length siderophore.

QS Repression of Amphi-enterobactin, DHBA, and DHB-Ser During Growth. To gain further insight into the production and use of amphi-enterobactin, DHBA, and DHB-Ser, we quantified these molecules during growth of wild-type (WT) cultures as well as a mutant strain that does not respond to QS (ΔQS) (14). Consistent with QS repression of siderophore production (14), concentrations of siderophores were much higher in the supernatants of the ΔQS mutant than in WT cultures (compare Fig. 4 A–C and Fig. 4 D–F). Time-resolved experiments using the ΔQS strain showed a steady increase in all three siderophores (Fig. 4 D–F). However, in WT cultures, amphi-enterobactin concentrations increased during exponential growth but decreased in stationary phase (Fig. 4A), whereas DHBA and DHB-Ser steadily accumulated (Fig. 4 B and C). As was the case for short-term experiments (Fig. 1 D–F), DHBA and DHB-Ser were both produced at much higher concentrations (micromolar) than amphi-enterobactin (nanomolar). The decrease in amphi-enterobactin in stationary phase provides direct evidence for amphi-enterobactin breakdown by V. harveyi (Fig. 4A). Here, repression by QS reduces the production rate of amphi-enterobactins below the breakdown rate. As a result, amphi-enterobactins are degraded in late growth stages, whereas soluble siderophores accumulate.

Untargeted LC-HR-MS/MS Search for Biosynthetic Intermediates. The Fe–enterobactin complex is exceedingly stable and Fe uptake requires the activity of esterases (33) that hydrolyze the siderophore. Suspecting that hydrolysis of amphi-enterobactins might contribute to the soluble siderophore pool in V. harveyi cultures, we used LC-HR-MS/MS to search for potential breakdown products of amphi-enterobactins in culture supernatants. Rather than look for all possible biosynthetic intermediates, we limited the search using our workflow for untargeted identification of iron-binding molecules (34). Specifically, searches for masses corresponding to the free ligand ([M+H]+), as well as the Fe and 54Fe complexes ([M-3H+56Fe]+) or [M-2H+56Fe]+ revealed several potential iron chelates. The corresponding apo-

McRose et al.
DHB-Ser, as well as other siderophore fragments form biologically available Fe complexes that are used by *V. harveyi* (Fig. 5A). Notably, Fe-uptake rates were nearly identical between spent medium and DHBA treatments, implying that DHBA may be responsible for most of the enhancement in Fe uptake.

There are two potential routes for Fe uptake from DHBA and DHB-Ser: direct transport of Fe-siderophore complexes and exchange of Fe between weaker siderophores and amphiphilic siderophobactins at the cell surface. Our data suggest that both processes occur. BLAST searches of the *V. harveyi* genome revealed hits to *CirA* (VIBHAR_01404 e-value of 7e-105) and *Fiu* (VIBHAR_06299, e-value of 3e-10), which transport DHB-Ser and other catechols (35, 37). In addition, comparisons of Fe-uptake rates in WT and ΔaebF *V. harveyi* hinted at Fe exchange. These experiments show that the presence of amphiphilic enterobactins aids in Fe uptake from soluble siderophores: Rates of Fe uptake from DHBA and spent medium were slower in mutants than in WT cultures (Fig. 5A). In contrast, mutant and WT strains showed no difference in Fe-uptake rate from Fe-EDTA buffered medium, suggesting that the mutant is only deficient in Fe uptake from soluble siderophores. Our experiments imply that amphiphilic enterobactins may be involved in some of the uptake of Fe from weaker siderophores (Fig. 5A), possibly through a ligand-exchange mechanism whereby Fe is transferred from weaker siderophores to amphiphilic siderophobactins at the cell surface.

Finally, we tested whether the concentrations of DHBA produced by WT cultures (1–2 μM) were tuned to the cellular Fe-uptake capacity. We measured Fe-uptake rates for a range of DHBA concentrations (1–10 μM). Fe-uptake rates increased with increasing concentrations of DHBA but saturated at concentrations greater than a few micromolar (Fig. 5B).

**Ecological Utility of Cell-Associated and Soluble Siderophores.** In the wild, *V. harveyi* has a complex lifestyle, existing as a free-living and a pathogenic microbe (38, 39). During growth, *V. harveyi* decreases concentrations of amphiphilic siderophobactins but accumulates soluble siderophores. This pattern of siderophore production—which is due to the breakdown of amphiphilic siderophobactins during uptake, decreased synthesis of amphiphilic siderophobactins due to QS, and what appears to be a diversity-oriented biosynthetic gene cluster (Figs. 6 and 7)—may provide beneficial versatility for different *V. harveyi* life stages.

It is of particular interest that *V. harveyi* adds a hydrophobic tail to enterobactin, a siderophore that is primarily produced by terrestrial species (40, 41). The increased solubility of charged molecules in high ionic strength seawater may necessitate the presence of a fatty-acid tail to anchor siderophores at the cell surface. The production of amphiphilic siderophores is in fact common among marine bacteria and has been proposed to reduce the loss of siderophores to diffusion in the dilute marine environment (42). During planktonic growth, *V. harveyi* must rely chiefly on amphiphilic enterobactins for Fe uptake as the bulk of DHBA and DHB-Ser will be lost to diffusion. The small size of amphiphilic enterobactins allows for tighter packing on the cell surface (compared with membrane-bound transporters) and could create an Fe “sink” at the cell surface. During growth in spatially confined environments (such as on particles, in biofilms, or during infections) where soluble siderophores can accumulate, *V. harveyi* may rely on DHBA and DHB-Ser for Fe uptake. Here, in addition to direct uptake from DHBA and DHB-Ser, Fe uptake could involve a “bucket brigade” mechanism (43–45) whereby Fe is shuttled by these soluble siderophores to amphiphilic siderophobactins at the cell surface (Fig. 5A).

**Fig. 3.** Amphiphilic enterobactin breakdown product (m/z = 527.2597) found in *V. harveyi* supernatant. (A) Fe-isotope pattern showing 54Fe/56Fe complexes as well as 13C isotopes. (B) MS/MS spectra and (C) proposed structure. See SI Appendix, Fig. S4 and Tables S1 and S2 for further breakdown products.

**Fig. 4.** Siderophore production over time. (A) Amphiphilic enterobactin m/z 965.3681, (B) DHBA, and (C) DHB-Ser production in WT cultures. (D) Amphiphilic enterobactin m/z 965.3681, (E) DHBA, and (F) DHB-Ser production in mutants that do not respond to QS (ΔQS, luxO D47E (14)). Error bars represent the standard deviation for biological duplicates.
Competition with other species is another reason for the production of both soluble and cell-associated siderophores. This has not been explored in *V. harveyi*, but competition assays using *E. coli* mutants that are deficient in enterobactin biosynthesis suggest that enterobactin is privatized at low cell density but shared at high cell density, and that this helps to protect against siderophore piracy (46). Our study supports the proposal of this previous work that enterobactin could be produced at low cell density while more soluble enterobactin derivatives or breakdown products (33, 35, 40) accumulate at high cell density. Measurements of amphi-enterobactin, DHBA, and DHB-Ser show exactly this type of transition in *V. harveyi*: Amphi-enterobactin concentrations increase during exponential growth but decrease during stationary phase, while the concentrations of DHBA and DHB-Ser continuously increase (Figs. 4A–C and 7). In some contexts, amphi-enterobactin may therefore function as siderophores available only to *V. harveyi*, whereas the weaker, more soluble DHBA and DHB-Ser are accessed by multiple bacteria.

**Repression by QS and Fe Optimizes Siderophore Production.** *V. harveyi* represses siderophore production when Fe levels are high. This result has been seen in many organisms and is a straightforward mechanism to avoid unnecessary secretion of siderophores. In *V. harveyi*, QS also represses siderophore production at high cell density. This may allow *V. harveyi* to devote resources to other processes such as growth and dispersal, as has been suggested in the case of QS repression of polymer secretion at high cell density in *Vibrio cholerae* (16). In addition, once cell density becomes sufficiently high, large per-cell siderophore production rates are simply no longer necessary to achieve maximal Fe uptake. It is the concentration of siderophores in the medium, not the per-cell production rate, that matters for Fe uptake. And, this process has limits: cellular Fe transport is not infinitely fast and cells do not have infinite surface area for siderophore transporters (47). The repression of siderophore production by QS may therefore allow *V. harveyi* to leverage “knowledge” of its population size to achieve a useful, but not profligate, siderophore concentration. This is seen in our experiments where increases in the concentrations of DHBA above ~2 μM (the concentration produced in stationary phase, Fig. 4), lead to increased Fe-uptake rates, but with diminishing returns (Fig. 5B). Indeed, the QS-dictated ~2 μM DHBA concentration seems well-tuned to the maximal uptake capacity of the cells.

QS regulates siderophores in species other than *V. harveyi*. For example, QS represses ornibactin in *Burkholderia cepacia* (11, 12) and vulnibactin in *Vibrio vulnificus* (13). In contrast, some *Pseudomonas aeruginosa* QS signals enhance the production of pyoverdine (15). In light of our proposal that increased siderophore production at high cell density is not beneficial for Fe uptake, we speculate that the direction of QS regulation may offer some broad insight into siderophore function. For example, in *P. aeruginosa*, siderophores are part of a strategy to accumulate high concentrations of Fe which trigger the production of biofilms and enable persistent infections (48). Siderophores repressed by QS may be primarily devoted to Fe acquisition for growth, while those stimulated by QS may have more complex roles, acting for example as signals or virulence factors or aiding in the establishment of chronic infections. This is certainly the case for pyoverdine (48, 49).

**Conclusion**

We have shown that *V. harveyi* has an Fe-and QS-repressed “two-for-one” siderophore gene cluster, that releases both cell-associated and soluble products. The most abundant of these soluble siderophores, DHBA and DHB-Ser, are found at concentrations nearly 100× higher than measured for amphi-enterobactin and are responsible for a large fraction of Fe uptake by *V. harveyi*. During planktonic growth, cell-associated siderophores may serve as transporters, allowing efficient Fe uptake from dilute marine environments. In contrast, during infections or growth on particles, where DHBA and DHB-Ser accumulate to high concentrations, these soluble siderophores become important for Fe uptake. Our results show that soluble and cell-associated siderophores may also work in concert. Finally, we find that QS repression of siderophore production allows...
V. harveyi to synthesize “just the right amount” of siderophores for Fe uptake. Our study is one of few to quantitatively investigate siderophore production in response to QS. Based on our findings, we make some more general predictions about the link between QS and siderophore production: namely that siderophores which are repressed by QS (as is the case in V. harveyi) are most likely used in Fe uptake for growth, whereas those stimulated by QS may serve other purposes acting for example as signals or virulence factors. Quantitative information about siderophore production and QS in other organisms will allow for the expansion, verification, and modification of these ideas.

Materials and Methods

Materials and methods including culturing conditions, BLAST searches, RNA extraction, RT-qPCR, siderophore quantification, LC-HR-MS/MS experiments, Fe-isotope studies, and Fe-uptake experiments are detailed in SI Appendix.

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