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Vibrio Iron Transport: Evolutionary Adaptation to Life in Multiple Environments

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SUMMARY

Iron is an essential element for *Vibrio* spp., but the acquisition of iron is complicated by its tendency to form insoluble ferric complexes in nature and its association with high-affinity iron-binding proteins in the host. Vibrios occupy a variety of different niches, and each of these niches presents particular challenges for acquiring sufficient iron. *Vibrio* species have evolved a wide array of iron transport systems that allow the bacteria to compete for this essential element in each of its habitats. These systems include the secretion and uptake of high-affinity iron-binding compounds (siderophores) as well as transport systems for iron bound to host complexes. Transporters for ferric and ferrous iron not complexed to siderophores are also common to *Vibrio* species. Some of the genes encoding these systems show evidence of horizontal transmission, and the ability to acquire and incorporate additional iron transport systems may have allowed *Vibrio* species to more rapidly adapt to new environmental niches. While too little iron prevents growth of the bacteria, too much can be lethal. The appropriate balance is maintained in vibrios through complex regulatory networks involving transcriptional repressors and activators and small RNAs (sRNAs) that act posttranscriptionally. Examination of the number and variety of iron transport systems found in *Vibrio* spp. offers insights into how this group of bacteria has adapted to such a wide range of habitats.

INTRODUCTION

Vibrionaceae are inhabitants of estuarine and marine environments worldwide (1). These Gram-negative bacteria are motile, facultative anaerobes that can grow with a number of different carbon and nitrogen sources. Vibrios may be found as free-living, planktonic cells; in biofilms; or colonizing a variety of marine organisms. Their metabolic adaptability and ability to occupy a variety of niches likely reflect their evolutionary history, which shows evidence of frequent gene acquisition. *Vibrio* species genomes are organized into two chromosomes (2). The majority of the essential genes map to the larger chromosome. The smaller

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chromosome has a mosaic structure with pathogenicity islands and phage-like genes, indicating numerous horizontal gene transfer events.

The vast majority of the >80 *Vibrio* species identified thus far are harmless to humans and marine organisms. However, some members of the species are associated with devastating epidemics (*Vibrio cholerae*) or sporadic infections (*Vibrio vulnificus* and *Vibrio parahaemolyticus*, among others). Human infections may follow the consumption of contaminated food or water or exposure of wounds to contaminated water. Because of their effects on human health, pathogenic vibrios have been extensively studied and are the focus of much of this discussion.

Vibrio spp., like most other organisms, have an absolute requirement for iron (3). Iron occurs in both the ferric (Fe^{3+}) and ferrous (Fe^{2+}) states and can participate in electron transfer over a wide range of redox potentials. Iron is essential for the function of a number of crucial enzymes in the bacterial cell (3), including ribonucleotide reductase for the synthesis of DNA precursors, cytochromes for electron transport, and tricarboxylic acid (TCA) cycle enzymes for energy production. In these enzymes, the iron cofactor may be heme, an iron-sulfur cluster, or, less often, an iron atom. The amount of iron required by vibrios varies depending on the cell's physiology and metabolism, but concentrations in the medium in the range of 0.1 μM to 5.0 μM provide sufficient iron for optimal growth of bacteria under laboratory conditions (4, 5). *V. cholerae* cells growing in broth medium at pH 7 with aeration will accumulate ~10^6 atoms of iron per cell (Carolyn Fisher, unpublished data), similar to amounts reported for *Escherichia coli* grown under similar conditions (6, 7).

Despite its abundance, iron is not readily available in most environments. The form and solubility of iron are strongly influenced by the E_h and pH of the environment and by the presence of anions that may form complexes with iron. Iron has low solubility in the presence of oxygen at neutral or slightly higher pH, where it is predominantly found as ferric hydroxides (8). The seawater environments that are home to the *Vibrionaceae* are slightly alkaline (pH 7.5 to 8.4), and the concentration of dissolved iron in the open ocean is usually in the nanomolar range but can be as low as 20 to 30 pM (9), well below the concentration required for most microorganisms. Iron is more abundant in coastal and estuarine areas due to runoff. The concentration of iron is lowest at the ocean surface (<0.2 nM) and is 4- to 5-fold higher at depths below 500 m. Some of the iron is released from oxygenated sediments on the continental margins and hydrothermal venting, but dust, particularly Saharan dust aerosol, is a major source of dissolved iron in the North Atlantic Ocean (10). In the ocean interior, remineralization from sinking particulate organic matter accounts for much of the soluble iron (10). The iron that is released at hydrothermal vents is converted to a bioavailable form by microbes present at the vents (11).

The low levels of available iron led Martin et al. (9) to suggest that iron is the growth-limiting factor in nutrient-rich ocean environments. Low iron levels not only reduce the rate of growth of bacteria but, in the case of vibrios, also affect their survival and ability to persist, particularly at the lower end of the ocean pH range (12). The addition of ferric oxide to water increased the survival of *V. cholerae*, even in the absence of nutrients (13). *Vibrio* spp. can enter a viable-but-nonculturable (VBNC) state under adverse conditions, such as low nutrient availability or cold (14, 15). Transcriptional analysis of genes upregulated by VBNC *V. cholerae* identified transport genes, including at least one iron transport system (16), further suggesting that iron acquisition is important for the survival of *V. cholerae* in the marine environment.

Although vibrios may be found as planktonic bacteria in ocean or estuarine waters, they are often found in biofilms (17), which can affect iron availability. Biofilms, in which the bacteria are embedded in an extracellular polysaccharide (EPS) matrix forming a complex three-dimensional structure, often form at solid-liquid or air-liquid interfaces. The effects of biofilm on iron availability are variable. Chelation of iron by negatively charged components of the EPS may increase the local concentration of iron. The amount of iron in *Vibrio* species EPS in nature is not known, but divalent cations are commonly found in biofilms (18). An analysis of pellicle biofilms isolated from mine drainage showed that iron accounted for ~70% of the cations present in the EPS. However, the binding of iron to EPS components may reduce its availability to bacteria. Reduced diffusion within biofilms can also lead to iron limitation, and the localized anoxic zones that occur in biofilms (19) influence the oxidation state and solubility of iron within these areas. Under low-oxygen, low-E_h conditions, iron is more likely to be in the ferrous than in the ferric form. Several bacterial species, including *Pseudomonas aeruginosa* (20) and *V. cholerae* (21), have reduced biofilm formation in low-iron environments, suggesting that biofilms are not conducive to iron acquisition.

Many vibrios form species-specific associations with marine organisms. *V. cholerae* can be found in the mucilaginous sheath of the filamentous cyanobacterium *Anabaena variabilis* (22), and this association allows the bacteria to persist longer in some environments. *Vibrio fischeri* (23) and *Vibrio logei* (24) (reclassified as *Aliivibrio* spp. [25]) form symbiotic relationships with squid, inhabiting the squid light organ. Extensive studies of the *V. fischeri*-squid association have shown not only that the bacteria colonize and produce light within the squid but also that the vibrios are required for normal development of the light organ (23, 26). Other vibrios colonize copepods, fish, or human hosts, and these interactions may range from relatively neutral to lethal for the host.

Colonization of a host presents additional challenges for microbial iron acquisition. In mammals, the level of freely available iron is far below that required for bacterial multiplication (27). The majority of iron is intracellular as heme, in iron-sulfur clusters, or stored as ferritin. Extracellular iron is bound to high-affinity iron-binding proteins, transferrin in serum and lactoferrin in secretions. Heme or hemoglobin released from cells is bound to hemopexin or haptoglobin, respectively. These proteins normally are relatively unsaturated and inhibit microbial growth by limiting iron availability. In response to infection, the level of available iron is further restricted by a reduction in serum iron saturation. This active withholding of iron from bacterial pathogens is a form of nutritional immunity (28) that protects against infection. In some human disease states, such as hemochromatosis or malaria, iron saturation increases, allowing the pathogen to obtain iron more easily and establish a more severe infection. In particular, *V. vulnificus* infections are more common in patients with iron overload.

Vibrios, like other bacterial species, have evolved a variety of mechanisms to acquire iron in each environment that they inhabit. Some of these iron acquisition systems are closely related among all vibrios, reflecting their common ancestry. Other trans-

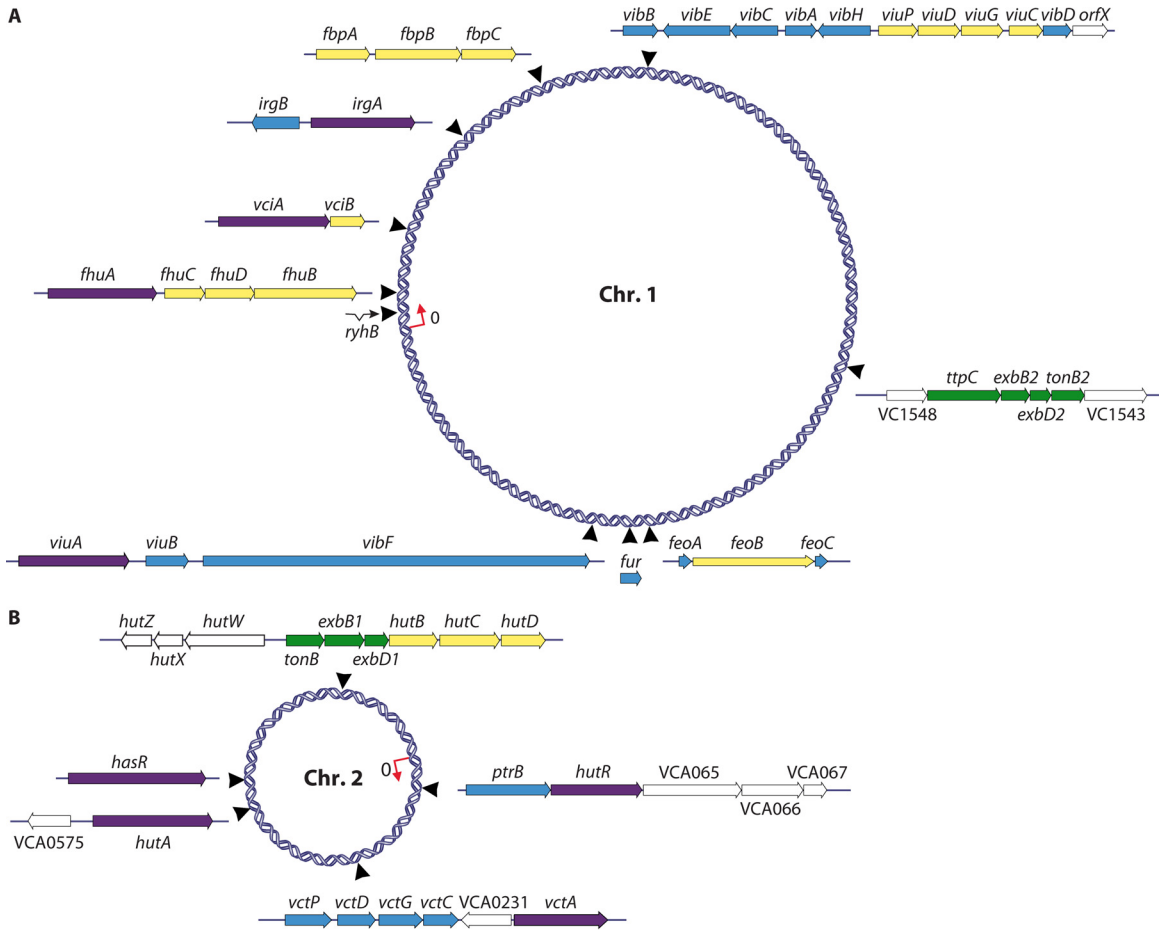

FIG 1 Map of *V. cholerae* chromosomes showing locations of known iron transport genes. Purple, outer membrane receptor genes; yellow, periplasmic binding protein and cytoplasmic membrane transporter genes; green, TonB system genes; blue, cytoplasmic protein genes. (Adapted from reference 31 [Fig. 1] with kind permission from Springer Science+Business Media.)

port systems appear to have been acquired by horizontal transfer and may indicate selective advantages in specific niches occupied by those *Vibrio* species. Iron transport system genes are found on both chromosomes in vibrios and encode transporters for ferric or ferrous iron, proteins for the synthesis and transport of high-affinity iron chelators, and specific receptors for iron proteins and various chelated forms of iron. The iron transport systems are best defined in *V. cholerae*, in which >1% of the genome is devoted to iron transport systems (Fig. 1). These iron transport systems, the genes encoding them, and the regulatory systems that control iron uptake have been described in detail. Thus, *V. cholerae* serves as a model for understanding iron acquisition in vibrios. What is known for the other *Vibrio* species and the similarities with and differences from *V. cholerae* are described below.

VIBRIO IRON TRANSPORT SYSTEMS

Transport of Iron Complexes

The majority of *Vibrio* iron transport systems recognize iron that is complexed to an iron chelator or carrier molecule (Fig. 2). The iron chelator may be synthesized by the bacteria or scavenged from other cells in the environment. *Vibrio* spp., like many other bacteria, produce one or more siderophores, small high-affinity ferric iron chelators (29). The siderophores are secreted into the environment or, less frequently, bound to the surface of the vibrio, and they serve as scavengers for iron. Once the siderophore binds iron, the ferrisiderophore complex is recognized by a specific receptor on the surface of the outer membrane (30, 31). Other iron complexes, including heme or siderophores made by other species (xenosiderophores), are also recognized by specific receptors. As described in more detail below, transport across the outer membrane via the receptor requires energy, which is supplied by the inner membrane protein TonB and its associated proteins ExbB and ExbD (32). Vibrios typically have more than one set of TonB/Exb proteins, and these proteins have specific as well as redundant functions (33). Once in the periplasm, the iron complex associates with a periplasmic binding protein (PBP) and is delivered to a cytoplasmic membrane permease for transport into the cytoplasm (34, 35). The periplasmic binding proteins and cytoplasmic permeases are less specific than the outer membrane receptors and recognize a broader class of complexes, usually additional members of the same class of siderophores. Once inside the bacterial cytoplasm, the iron is released from the siderophore by reduction of the iron and/or enzymatic cleavage of the siderophore, making the iron available for cellular needs (36, 37).

***Vibrio* siderophores.** Each of the *Vibrio* species analyzed thus far produces one or more siderophores when iron is limiting.

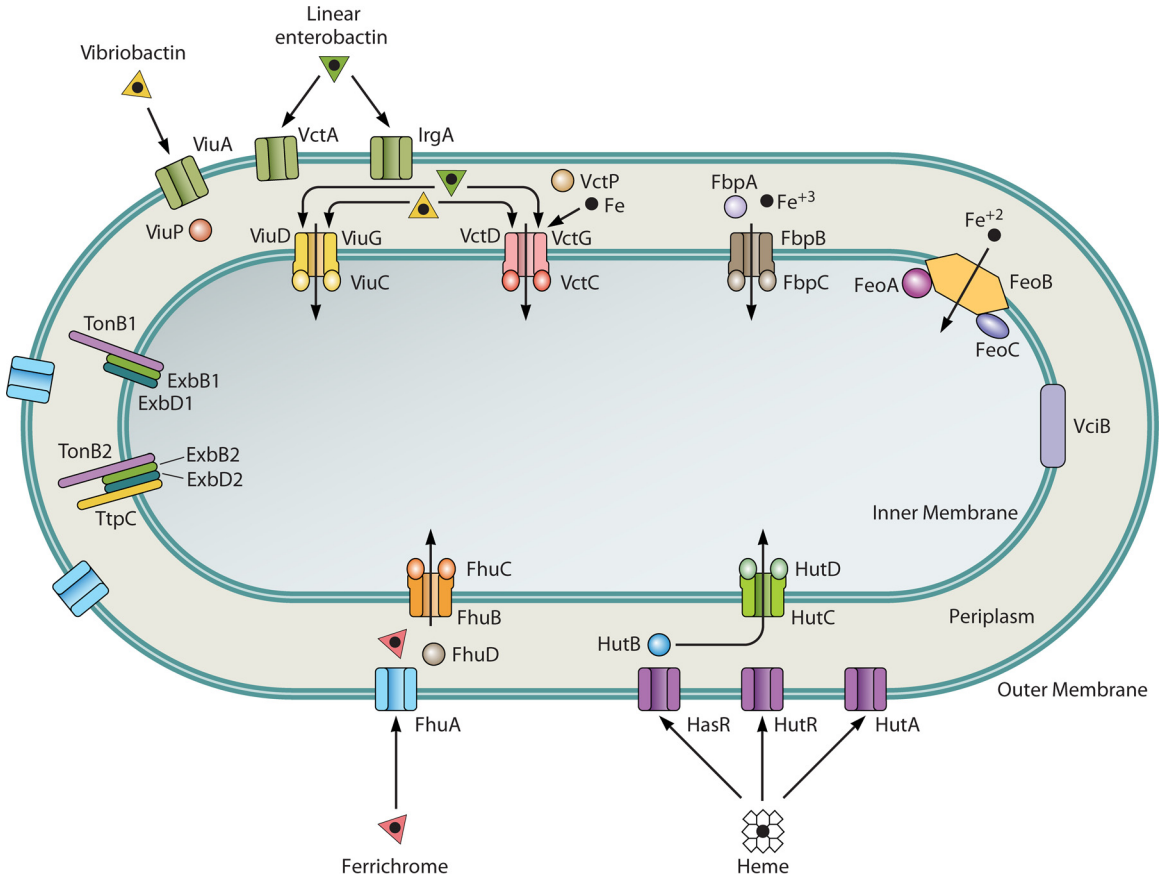

FIG 2 *V. cholerae* iron transport systems. The cell envelope locations of components of the major iron transport systems and the compounds transported through each system are shown. (Adapted from reference 154 with permission of the publisher [copyright 2011 Blackwell Publishing Ltd.].)

These molecules often have catechols or hydroxamates as part of the iron-binding moieties, although other structures have been identified. In some species, such as the squid symbiont *V. fischeri*, the structure of the siderophore is unknown (38), but a number of vibrio siderophores have been isolated and characterized, and there is considerable diversity in their structure and chemical properties. As described in more detail in the discussion of individual siderophores below, this diversity is likely a reflection of vibrios evolving to grow in different environments and to produce siderophores that can outcompete those produced by other members of the community when iron is scarce.

(i) Catechol siderophores. Catechol siderophores, which have 2,3-dihydroxybenzoic acid (DHBA) as the iron-chelating moiety, are common among *Vibrio* spp. Catechols and their biosynthesis were first described in *Enterobacteriaceae*, and little variation in catechol structures has been noted for this group of bacteria. *Escherichia coli* (39), *Salmonella* (40), and most *Shigella* spp. (41, 42) produce enterobactin, a cyclic trimer of 2,3-dihydroxybenzoylserine (Fig. 3A). The notable example of variation in catechol structure in this group is the salmochelins produced by some pathogenic *Enterobacteriaceae*, in which the 2,3-dihydroxybenzoylserine is glucosylated (43). In contrast, vibrios have a much more varied repertoire of catechols. The vibrio catechols are generally linear structures that differ in their backbones and in the numbers and types of amino acids linked to the catechols.

(a) Vibriobactin. V. cholerae produces the catechol vibriobactin, which has three catechol groups attached to a norspermidine backbone (44) (Fig. 3A). The genes for norspermidine synthesis are not commonly found in bacteria but are widely distributed in *Vibrio* spp. (45). Two of the DHBA molecules are linked to the backbone through threonines that are cyclized, forming oxazoline rings. The vibriobactin biosynthesis mechanisms and pathway were determined by the Walsh group (46–49) (Fig. 4A). VibF, a nonribosomal peptide synthetase (NRPS), together with the vibriobactin synthase proteins VibE, VibH, and VibB assemble the siderophore from its precursors DHBA, threonine, and norspermidine, (46). DHBA is the product of VibA, VibB, and VibC (50), which have the same functions as their *E. coli* homologs EntA, EntB, and EntC (51). VibF acts as the scaffold on which vibriobactin synthesis is completed and has six domains organized in an assembly-line fashion (Fig. 4B). There are two cyclization domains, followed by an adenylation domain, a condensation domain, a peptidyl carrier protein (PCP) domain, and a second condensation domain (46). This assembly process on a NRPS with distinct, modular domains is characteristic of bacterial catechol siderophores and other secondary metabolites, including antibiotics, and has been described in detail for *E. coli* and other species (52–54). The domain structure of the NRPSs has facilitated the evolution of a large family of distinct siderophores with variations in the peptide backbones, chelating moieties, or other modifications. There is considerable homology among the NRPSs, but dif-

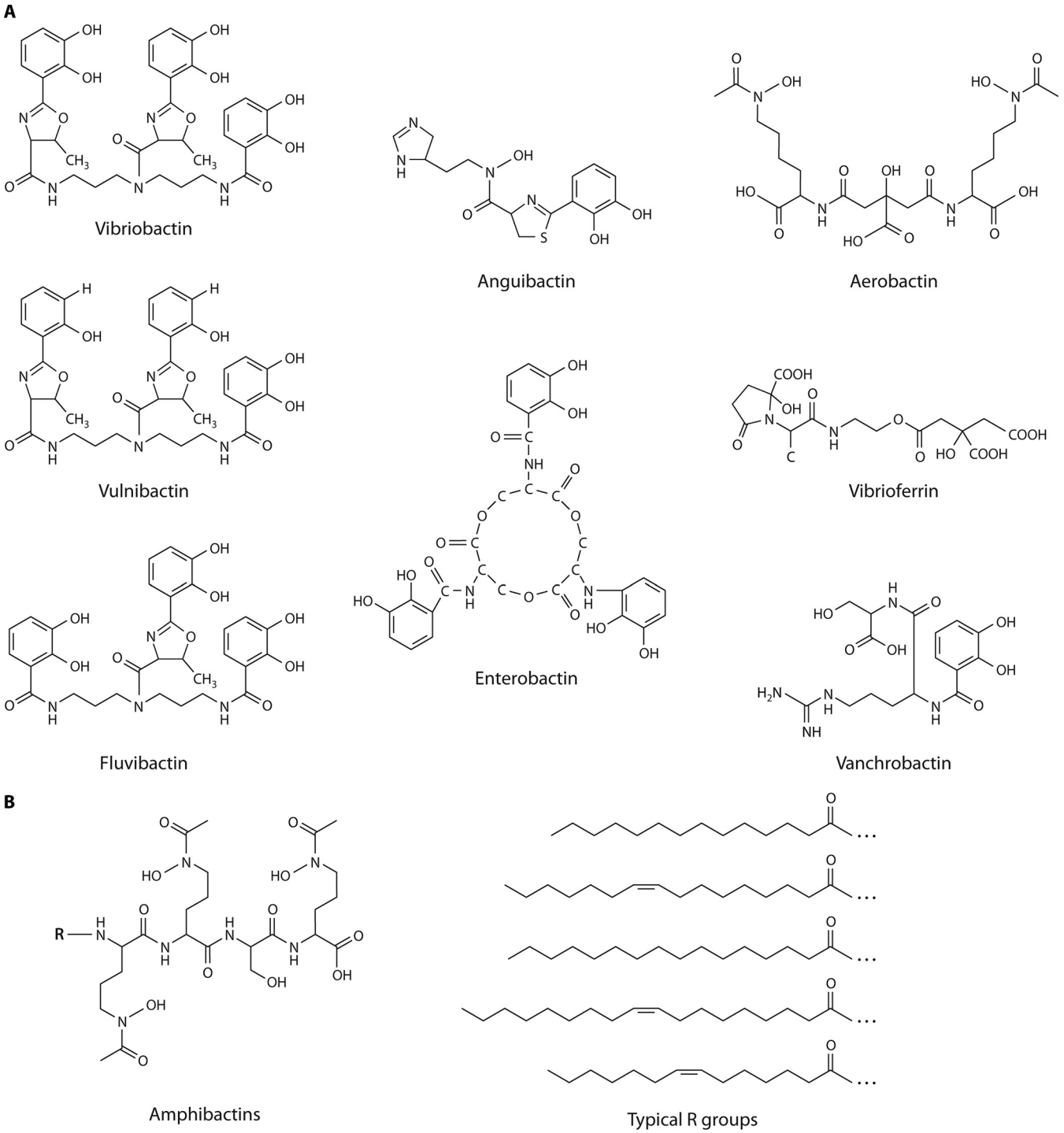

FIG 3 Structures of representative vibrio siderophores. (A) Catechol and carboxylate siderophores. (B) Amphibactins.

ferences in the numbers of domains and in their substrates are evident. Duplications or substitutions of domains allow different amino acids or chelating groups to be incorporated into the final structure, resulting in a family of related, but distinct, siderophores produced by different *Vibrio* species.

The genes encoding vibriobactin synthesis are found in two separate clusters on the large chromosome (Fig. 1). The genes for the synthesis of the catechol moiety, *vibABC*, are linked to genes for subsequent steps in biosynthesis, *vibD*, *vibE*, and *vibH*, and to genes for catechol transport, *viuPDGC* (50). The NRPS VibF is encoded by a second vibriobactin gene cluster on the large chromosome linked to genes for vibriobactin transport, *viuA*, and utilization, *viuB* (55) (Fig. 1). It is not uncommon in vibrios for the core catechol genes to be found as one module and one or more

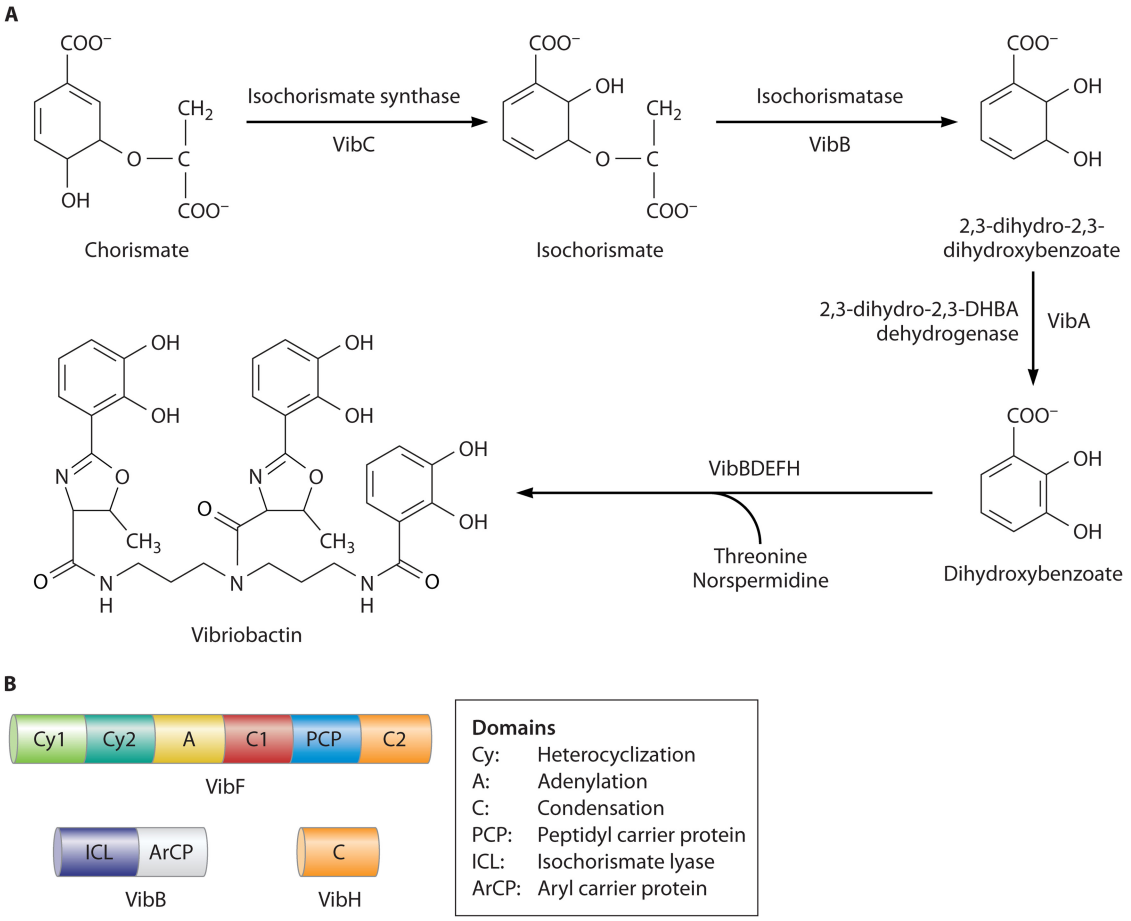

FIG 4 Vibriobactin biosynthesis. (A) Biosynthetic pathway. 2,3-Dihydroxybenzoate is synthesized from chorismate by the sequential activities of VibC, VibB, and VibA. VibB is a bifunctional enzyme that is also required for a later step in biosynthesis. VibB and VibF are modified by the attachment of phosphopantetheine arms to the aryl carrier protein domain of VibB and the peptidyl carrier domain of VibF (46). VibD, which is required for late steps in vibriobactin synthesis (263), is predicted to be the phosphopantetheine transferase based on homology and its ability to complement an *E. coli entD* mutation (50). VibE activates DHBA forming the acyl adenylate and transfers it to the free thiol of the phosphopantetheine (47). This DHB thioester is combined with norspermidine by the condensation domain protein VibH to produce N^1-(2,3-dihydroxybenzoyl)norspermidine (47). VibF activates and covalently loads the PCP domain with L-threonine and heterocyclizes 2,3-dihydroxybenzoyl-VibB with L-Thr. The aryl oxazoline is then transferred to N^1-(2,3-dihydroxybenzoyl)norspermidine. VibF catalyzes a second oxazoline acylation, yielding vibriobactin. (B) Domain structure of the vibriobactin biosynthesis enzymes VibF, VibB, and VibH. The NRPS VibF is the scaffold for assembly, and the indicated domains act in an assembly-line fashion to construct the final product.

genes encoding the species-specific modifications occurring elsewhere in the genome as a second module.

(b) Fluvibactin and vulnibactin. Vibrio fluvialis and *Vibrio vulnificus* produce catechol siderophores, fluvibactin and vulnibactin, that are structurally related to vibriobactin (56, 57) (Fig. 3A). Like vibriobactin, these siderophores have a linear, norspermidine backbone with the iron-binding moieties linked directly or through threonine to the backbone. In fluvibactin, however, only one of the three DHBA moieties is linked to norspermidine by forming an oxazoline ring with threonine, and the other two are linked directly to norspermidine. Vulnibactin represents another variation on the theme: it has a norspermidine backbone with one catechol linked directly to the backbone, but it differs in having two salicylate residues, rather than DHBA, forming the oxazoline rings with threonine (56) (Fig. 3A). A mutant of *V. vulnificus* defective in salicylate biosynthesis was rescued for growth in low iron by the addition of DHBA, suggesting that *V. vulnificus* may also be able to produce a siderophore having DHBA instead of salicylate (58). The genes encoding vulnibactin have not been fully characterized, but the identification of *venB* (isochorismate lyase) (59) and two AMP ligase genes (58) within a cluster of homologs of known NRPSs suggests that a region on chromosome 2 of *V. vulnificus* (VV2_0828 to VV2_0844) encodes vulnibactin biosynthesis.

(c) Anguibactin, a mixed catechol and hydroxamate siderophore. The *V. anguillarum* O1 serotype, a fish pathogen, also produces a catechol siderophore, but its structure is distinct in containing both catechol and hydroxamate groups as part of the iron-binding residues (60) (Fig. 3A). This siderophore, anguibactin, is synthesized from DHBA, which provides the catechol group; *N*-hydroxyhistamine, which contributes the *N*-hydroxyl group characteristic of hydroxamate siderophores; and L-cysteine (61–63).

Most of the genes required for anguibactin synthesis are carried on a 65-kb plasmid, but there is some redundancy with chromosomal genes. Genes for DHBA synthesis (*angABC*) and activation (*angE*) are chromosomal (64), but the NRPS domain proteins are encoded on the plasmid. The plasmid has homologs of the *angB*, *angC*, and *angE* genes, but there is not an *angA* equivalent

among the plasmid-borne genes. Some of the plasmid-borne genes are flanked by insertion sequences, forming a composite transposon-like structure (65, 66). This suggests that *V. anguillarum* acquired the anguibactin iron transport system by horizontal transmission. Homologs of the plasmid genes are present in the chromosome of *Vibrio harveyi*, which also produces anguibactin, further supporting horizontal transfer of the genes (67).

V. anguillarum O1 strains that lack the anguibactin plasmid, as well as strains of a number of other *V. anguillarum* serotypes, synthesize vanchrobactin (Fig. 3A), a catechol siderophore distinct from anguibactin (68–70). Vanchrobactin is a linear dipeptide derived from arginine and serine bound to DHBA (71), indicating some similarity to the enteric siderophore enterobactin (69).

(ii) Carboxylate siderophore. *Vibrio parahaemolyticus* produces a very hydrophilic carboxylate siderophore that is unrelated to other characterized *Vibrio* siderophores. This compound, given the trivial name vibrioferrin, is composed of alanine, citric acid, ethanolamine, and 2-ketoglutarate (72) (Fig. 3A). Vibrioferrin has a lower affinity for iron than do the *Vibrio* catechol siderophores. In addition, it is more sensitive to photolysis than other photoreactive siderophores, resulting in the release of iron even under low-light conditions (73). Although the instability and low iron affinity of vibrioferrin would appear to be disadvantageous, studies of another Gram-negative marine bacterium, *Marinobacter*, offer insights into a unique role for vibrioferrin. Vibrioferrin has been isolated from alga-associated subclades of *Marinobacter*, and it was proposed that its low affinity and photoreactivity are evolutionary adaptations to the bacterium-alga mutualism (74). The phototactic dinoflagellates form aggregates with *Marinobacter* at the sea surface, where sunlight promotes photolysis of the vibrioferrin secreted by the bacteria. This results in a local increase in the concentration of uncomplexed ferric iron that can be used by both *Marinobacter* and the dinoflagellates. The iron allows the algae to increase the amount of fixed carbon, some of which is made available to the bacteria (74, 75). *V. parahaemolyticus*, like *Marinobacter*, can associate with marine algae (76), and it is possible that mutualistic interactions involving vibrioferrin occur with *V. parahaemolyticus* as well.

(iii) Hydroxamate siderophores. Although many of the siderophores produced by vibrios are catechols, hydroxamate compounds have also been found in some species. Aerobactin, a hydroxamate siderophore found in many enteric bacteria, including *E. coli*, *Shigella*, and *Salmonella*, is also produced by some *Vibrio* species. *Vibrio mimicus* (77), *Vibrio hollisae* (78), and a planktonic marine *Vibrio* sp. (79) all produce this siderophore. Interestingly, the genes for aerobactin synthesis and transport (*iucABCD* and *iutA*) in *V. mimicus* and *V. hollisae* have the same arrangement as that in the enteric species, and the proteins show considerable identity to the corresponding *E. coli* proteins (78). In the *Enterobacteriaceae*, the genes are found on plasmids (80, 81) and in pathogenicity islands (82–85). Thus, it is likely that these genes have spread among the various bacterial strains by horizontal transmission. A strain of *Vibrio harveyi* isolated from the open ocean of the Gulf of Mannar produced a dihydroxamate siderophore (86). However, its complete structure has not been determined. *V. vulnificus* also produces a hydroxamate siderophore (87) in addition to its catechol vulnibactin, but the exact structure of the hydroxamate is not known.

(iv) Amphiphilic siderophores. Many marine bacteria have been found to produce amphiphilic siderophores, which have a hydrophobic tail attached to the hydrophilic siderophore backbone. These siderophores are often produced by the bacteria as a suite of related compounds with a conserved ferric iron-binding peptide or citrate-based head group and one or two fatty acids (88–90). The fatty acid appendages may vary in length and the degree of saturation and hydroxylation. Depending on the peptidic head group and length of the acyl chain, these siderophores may be predominantly cell associated or more water soluble and secreted into the environment (90). Both types of siderophores, termed amphibactins, have been isolated from marine *Vibrio* species. *Vibrio* species strain HC0601C5 produces a hydrophilic suite of amphibactins that are primarily secreted into the medium (90), while *Vibrio* sp. strain R-10 produces amphibactins that are cell associated (89) (Fig. 3B). The amphibactins have a hydroxamate-containing head group composed of a serine and three ornithine residues with acyl groups ranging from C_{12} to C_{18}. The moanachelins, produced by *Vibrio* sp. strain Nt1, are related to the amphibactins but differ in the head group, in which the serine is replaced by glycine or alanine (91). *V. harveyi* produces an amphiphilic variant of enterobactin, the catechol siderophore produced by most *Enterobacteriaceae* (92). *V. harveyi* has a cluster of genes homologous to the *E. coli ent* genes with a long-chain fatty acid coenzyme A (CoA) ligase gene located nearby. The structure of the siderophore is similar to that of Ent but has an acyl-L-serine in addition to the three dihydroxybenzoyl-serines that make the cyclic backbone of Ent. Like the other amphiphilic siderophores produced by *Vibrio* spp., amphi-enterobactin is produced as a family of siderophores differing in the acyl chain (92). The production of a collection of these compounds by a *Vibrio* species may result in the less-hydrophobic siderophores being released into the environment, while the more hydrophobic members of the suite would remain cell associated. It was proposed that this would create a gradient of iron chelators extending from the bacterium and would allow the iron to be passed from the secreted siderophores to those at the surface (89). How the iron transits from the membrane-associated siderophore to the interior of the cell is not known, but hydrolysis of the fatty acids could release the membrane-bound form of the siderophore for its transport into the cell (93). An amide hydrolase produced by a *Marinobacter* species strain in late log phase hydrolyzed the *Marinobacter* amphiphilic siderophores. In addition, when *Marinobacter* sp. and *Halomonas aquamarina* were cocultured, the *Marinobacter* enzyme hydrolyzed the suite of aquachelins produced by *Halomonas*, even though these aquachelins have a different peptidic head group (93). Thus, enzymatic modifications of secreted or cell surface-associated siderophores may occur in the aquatic environment, where a variety of bacterial species coexist.

Siderophore receptors. The fully assembled siderophore is secreted from the cell or localized to the outer surface, where it binds ferric iron from the extracellular environment. Binding results in a conformational change in the siderophore that in vibriobactin is evidenced by the change in color of the siderophore from colorless to deep purple when the siderophore is complexed to ferric iron (44). This iron-siderophore complex cannot diffuse back into the cell but requires a specific outer membrane receptor (37, 94–96) (Fig. 2). These receptors belong to the TonB-dependent receptor family and require TonB to energize transport. Each *Vibrio* species produces a receptor specific for its siderophore, and the receptor gene is usually linked to genes encoding the siderophore biosyn-

TABLE 1 Siderophore synthesis and use by selected *Vibrio* species

Species and siderophore	Reference(s)
V. cholerae	
Endogenous siderophore	
Vibriobactin	44, 46
Xenosiderophores	
Linear enterobactin derivatives	44, 106, 108
Ferrichrome	44, 107
V. vulnificus	
Endogenous siderophores	
Vulnibactin	56
Uncharacterized hydroxamate	87, 186
Xenosiderophores	
Aerobactin	257
Deferoxamine B	111, 112
Vibriobactin	117
V. parahaemolyticus	
Endogenous siderophore	
Vibrioferrin	72
Xenosiderophores	
Enterobactin	258, 259
Aerobactin	109
Ferrichrome	110
Vibriobactin(?)	117
Fluvibactin(?)	117
V. anguillarum	
Endogenous siderophores	
Vanchrobactin (strains lacking plasmid pJM1)	68–70
Anguibactin (strains with plasmid pJM1)	60
Xenosiderophores	
Enterobactin	260
Rhodotorulic acid	261
Ferrichrome	261
Citrate	262

thesis enzymes. *V. cholerae* transports vibriobactin via the outer membrane receptor ViuA (94). Similarly, PvuA is the vibrioferrin receptor in *V. parahaemolyticus* (97), VuuA is the vulnibactin receptor in *V. vulnificus* (98), and FvtA transports vanchrobactin in *V. anguillarum* (99). The siderophore receptors are typically localized to the outer membrane without the need for accessory factors. However, the *V. anguillarum* anguibactin receptor FatA (100, 101) requires O-antigen side-chain biosynthesis for outer membrane localization (102). The phenotype of the O-antigen mutants was similar to that of a *fatA* mutant. This indicates a novel role for O-antigen in the localization or stability of the siderophore receptor in *V. anguillarum*.

Xenosiderophore receptors. Cheating, or at least taking advantage of the neighbors, is a common phenomenon in iron transport. Most bacteria express one or more receptors for siderophores that they do not themselves synthesize, allowing them to acquire iron bound to siderophores made by other species. Examples of the variety of siderophores transported by *Vibrio* spp. are shown in Table 1. Because vibrios live in polymicrobial habitats, there may be strong evolutionary pressure to acquire xenosiderophore transport systems. Iron is a limited resource, and the secreted siderophores that can deliver this resource represent a common good. A siderophore secreted by one species would promote the growth of other members of that species and simultaneously sequester iron from unrelated species that are unable to use the siderophore. Therefore, the production of species-specific siderophores represents a form of kin discrimination (103). Members of other species bypass this kin discrimination by the acquisition of receptors allowing the recognition and transport of the xenosiderophore. *Vibrio* spp. exhibit natural competence and readily acquire new genes by transformation (104, 105). Such horizontal gene transfer events appear to be responsible for many of the genes encoding xenosiderophore transporters.

V. cholerae has receptors for nonnative catechol siderophores (106) and for ferrichrome, a fungal hydroxamate siderophore (107). The *V. cholerae* catechol receptors VctA and IrgA recognize linear derivatives of enterobactin, which is produced by *E. coli* and other enteric bacteria, while fluvibactin can be transported by either of these receptors or by ViuA (108). *V. parahaemolyticus* can use aerobactin (109) and ferrichrome (110). Both *V. vulnificus* (111, 112) and *V. furnissii* (113) have a receptor, DesA, for the hydroxamate deferoxamine B. *desA* expression requires a positive transcription factor, DesR. This AraC-like regulator promotes the transcription of *desA* in the presence of deferoxamine B (113). Thus, the expression of this system is induced when the siderophore is present in the environment.

Not surprisingly, some bacteria have evolved mechanisms to outwit the cheaters and scavengers. This can be seen in studies of polymicrobial communities in which some bacteria produce siderophores that not only cannot be used by competitors but also inhibit the competition. For example, the addition of the siderophore produced by a potential fish probiont, *Pseudomonas fluorescens* strain AH2, to a growing culture of *V. anguillarum* caused immediate growth arrest (114). Growth was restored by the addition of iron, suggesting that the *P. fluorescens* siderophore outcompetes *V. anguillarum* for iron, resulting in iron starvation. Further analysis of the *V. anguillarum* response to the antagonistic siderophore showed the induction of *rpoS*, encoding the alternative sigma factor associated with the stationary phase and the stress response. Thus, *P. fluorescens* may protect fish from infection with *V. anguillarum* by producing an antagonistic siderophore that withholds iron and induces stationary-phase-like growth arrest in the vibrios. Similarly, *Shewanella algae* can inhibit *V. alginolyticus*. *V. alginolyticus* has at least a dozen genes encoding putative siderophore receptors (115) and can use ferrichrome (116) and siderophores secreted by *V. cholerae*, *V. fluvialis*, and *V. parahaemolyticus*, in addition to its own siderophore (117). However, avaroferrin, a cyclic dihydroxamate siderophore secreted by *S. algae* isolated from the same red seaweed as *V. alginolyticus*, inhibited *V. alginolyticus*. Swarming of *V. alginolyticus* was inhibited at low concentrations of the siderophore, and this inhibition was greater than that observed with other xenosiderophores (115). Structural analysis of avaroferrin indicates that it is a chimera of two known siderophores, bisucaberin and putrebactin. The chimeric nature of the siderophore is reflected in the genetics of avaroferrin biosynthesis genes. The biosynthesis genes have homologs in the *Vibrio salmonicida* (bisucaberin) and *Shewanella putrefaciens* (putrebactin) siderophore biosynthesis gene clusters, and Böttcher and Clardy (115) point out that the modular nature of siderophore biosynthesis genes may favor recombination and the evolution of new siderophores. In this case, the chimeric siderophore can be used by the species that synthesizes it but is inhibitory to other bacteria, at least until they acquire the genes

necessary for the use of avaroferrin. Thus, the evolutionary arms race for iron continues.

Heme receptors. In addition to the siderophore receptors, vibrios have one or more receptors for the transport of heme and can use heme for growth in low-iron environments. These receptors are also members of the TonB-dependent transporter family and have homology to the siderophore receptors. *V. cholerae* has three heme receptors, designated HutA, HutR, and HasR (118–120). HutA and HutR share significant homology, while HasR more closely resembles the HasR hemophore-dependent heme transporters in *Pseudomonas aeruginosa* and *Serratia marcescens* (120). The hemophore-dependent receptors in *P. aeruginosa* and *S. marcescens* bind heme complexed to a heme carrier protein, the hemophore (121), but no hemophores have been identified in *V. cholerae* or other *Vibrio* species. Each of these receptors allows growth in medium containing heme as the sole iron source. Heme is transported into the cell as an intact iron-porphyrin complex and supports the growth of a heme biosynthesis mutant (118). It is not known whether the heme is used by *V. cholerae* solely as an intact heme complex or if it is broken down following transport to release the iron. A candidate for a heme oxygenase-like enzyme to degrade heme is the cytoplasmic protein HutZ, which is needed for the efficient use of heme as an iron source in *V. cholerae* (122). An analysis of HutZ *in vitro* indicated that it has a high affinity for heme and degrades it via the same intermediates as heme oxygenase (123). Thus, HutZ may serve to release iron from heme following transport.

Heme receptors in other species include HupA (124), HvtA (125), and HupO in *V. fluvialis* (126); HuvA in *V. anguillarum* (127, 128); and MhuA in *V. mimicus* (129). HvtA has 68% similarity and 51% identity to *V. cholerae* HutR, and the operon organization is the same in the two species, indicating a close genetic relationship (120, 125). Although *V. cholerae* has only a limited ability to use hemoglobin as an iron source, other *Vibrio* species, including *V. parahaemolyticus*, *V. fluvialis* (126), *V. alginolyticus* (130), *V. anguillarum* (131), and *V. vulnificus* (132, 133), have the ability to use hemoglobin efficiently. This is dependent on specific receptors that bind hemoglobin or proteases that can release the heme from heme proteins. In *V. vulnificus*, for example, both HupA and HvtA can transport hemin, but only HupA permits the use of hemoglobin (125). *V. vulnificus* can use hemoglobin even when it is bound to the hemoglobin-binding protein haptoglobin (133, 134). Hemoglobin can also be bound by albumin. The ability of *V. vulnificus* to obtain heme from the hemoglobin-albumin complex was associated with the production of an extracellular protease that liberated heme from the proteins (134).

TonB Systems in *Vibrio* Iron Transport

The transport of siderophores and heme across the outer membrane of *Vibrio* requires energy. This is problematic since there is no ATP or other potential energy source available to transporters in the outer membrane. *Vibrio* spp. and other Gram-negative bacteria solve this problem by coupling outer membrane receptors for iron sources to the cytoplasmic membrane proton motive force. The TonB-ExbB-ExbD complex that is embedded in the cytoplasmic membrane of Gram-negative bacteria transduces the energy across the periplasm to the outer membrane receptors (32) (Fig. 5). Interestingly, the vibrios have more than one set of TonB/Exb proteins. *V. cholerae* (33, 120, 135), *V. anguillarum* (136), and *V. alginolyticus* (116) have two TonB systems, and *V. vulnificus* (137) has three. These systems differ in their recognition of specific receptors and in the environments in which they function, as discussed below. In addition to the Exb proteins, some *Vibrio* TonB systems also include an additional factor, TtpC (138–140). All of the *Vibrio tonB2* gene clusters, as well as some *tonB3* operons, include a *ttpC* gene (139). TtpC is a cytoplasmic membrane protein that is predicted to have three membrane-spanning domains with its C terminus in the cytosol and its N terminus located within the periplasm (140). TtpC is part of the TonB2-ExbB2-ExbD2 complex and is required for the transport of heme or siderophores that use the cognate outer membrane receptors (140).

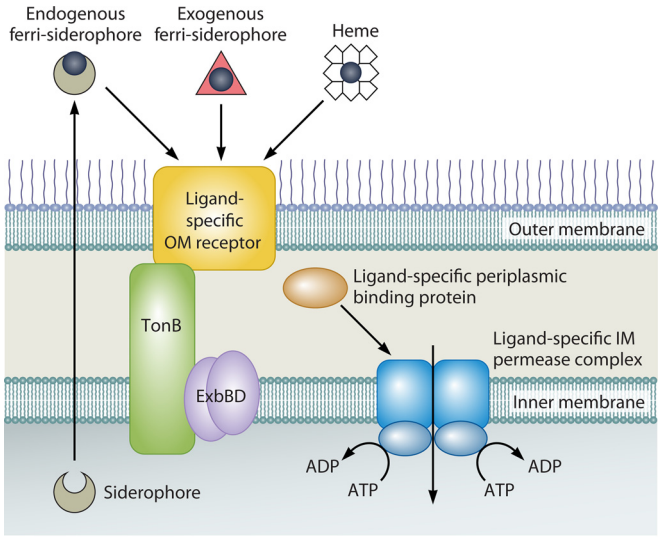

FIG 5 General scheme for TonB-dependent iron transport systems. The iron complex is recognized by a specific receptor in the outer membrane (OM). *Vibrio* spp. have receptors for their own endogenously synthesized siderophores and for exogenous siderophores produced by siblings or by members of other species (xenosiderophores). Transport across the outer membrane is dependent on energy provided by the TonB-ExbB-ExbD complex. Following transport, the siderophore or heme is bound to a periplasmic binding protein and is delivered to the ATP-dependent inner membrane (IM) permease.

The first TonB system characterized in *Vibrio*, the TonB1-ExbB1-ExbD1 system, is encoded on the smaller of the two chromosomes of *V. cholerae*, and the genes are in an operon containing genes for the use of heme as an iron source (33) (Fig. 1). A second system, the TonB2-ExbB2-ExbD2 system, maps to the larger chromosome (33) (Fig. 1). Analysis of the TonB1 and TonB2 systems indicates that they have overlapping functions but are not fully redundant. Vibriobactin and ferrichrome are transported efficiently using either TonB1 or TonB2 (33, 135). Either TonB system also allowed heme transport via HutA and HutR, although transport was more efficient in strains carrying the *tonB1* system genes (120). In contrast, heme uptake via HasR was TonB2 dependent (120). The TonB2 system is required for catechol transport via the VctA and IrgA receptors in *V. cholerae* (106), and *tonB2*, but not *tonB1*, can complement *E. coli tonB* mutations for transport of the catechol siderophore enterobactin (33). It was shown through characterization of chimeric TonB molecules, as well as analyses of TonB1 point mutants, that the specificity of TonB1 for outer membrane receptors in *V. cholerae* resides within the carboxy terminus of TonB1 (141). Conversely, the ability of a receptor to be energized by TonB1 is determined by a single amino acid residue in the N-terminal domain of these receptors (141).

TonB1 interacts with a restricted group of *V. cholerae* receptors but has the ability to function under environmental conditions, i.e., growth with high salt levels, that do not allow TonB2 to support transport through some receptors (135). TonB1 is 38 amino acids longer than TonB2, and most of this extension maps to the proline-rich region that spans the periplasm. Under conditions of increased osmolarity, such as those of seawater, which increase the periplasmic space (142), the longer TonB1 system could span the periplasm to interact with the receptors. The shorter TonB2 system does not function with a subset of the outer membrane receptors, including the heme receptors, in medium with increased salt concentrations. Deletion of 35 amino acids of the periplasmic domain region of TonB1 did not affect its ability to function at low osmolarity but eliminated its interaction with the heme receptors at higher osmolarity (135). Thus, TonB1 may specifically be required for transport through a subset of TonB-dependent receptors when *V. cholerae* is in the marine environment, while TonB2 is required for the use of some catechol xenosiderophores.

V. alginolyticus also has two TonB systems that have distinct functions. While both the TonB1 and TonB2 systems supported the use of vibrioferrin and the xenosiderophore ferrichrome, TonB1 was specifically required for heme use (116).

Transport from the Periplasm to the Cytoplasm

Following transport across the outer membrane, the siderophore or heme is bound by a periplasmic binding protein (PBP) and handed off to an ATP-binding cassette (ABC) transporter for transit across the cytoplasmic membrane. The periplasmic and cytoplasmic membrane proteins recognize specific ligands but tend to be less discriminating than the outer membrane receptors. Thus, vibriobactin is recognized by the outer membrane receptor ViuA (94) and not by the catechol receptors VctA and IrgA (106), but once in the periplasm, either of the catechol PBP and ABC transporters VctPDGC and ViuPDGC can transport the siderophore into the cell (106). Similarly, enterobactin derivatives are not recognized by ViuA but can be transported by both the Vct and Viu periplasmic and cytoplasmic membrane transport systems (96).

The transport of hydroxamate siderophores has been characterized in less detail than catechol transport in the *Vibrionaceae*. Hydroxamate siderophores also have specific outer membrane receptors but often share periplasmic binding proteins and cytoplasmic transporters (143). In *V. parahaemolyticus*, ferrichrome and aerobactin have distinct receptors, but FhuBCD transports both of these hydroxamates through the periplasm and across the cytoplasmic membrane (110).

Once in the cytoplasm, iron must be removed from the siderophore for use by the cell. This can be accomplished by the reduction of ferric iron to ferrous iron, for which the siderophores have low affinity, or by enzymatic cleavage of the siderophore. The use of vibriobactin-bound iron requires ViuB (37), and based on its homology to the *E. coli* siderophore-interacting protein YqjH (144), it is likely to act as a ferric reductase and to release iron from vibriobactin. Although *E. coli* uses an esterase, Fes, to break down enterobactin to release iron (36), no homolog of Fes is found in *V. cholerae*. Because *V. cholerae* uses linear rather than cyclic catechols, it may not be necessary to degrade the siderophore to release the iron (108). Other *Vibrio* spp., however, have Fes homologs, including *V. parahaemolyticus* (75% amino acid sequence identity), *V. alginolyticus* (37% identity), and *V. anguillarum* (36% identity). The *V. anguillarum* Fes homolog VabH has been partially characterized and was shown to be required for the use of the *V. anguillarum* siderophore vanchrobactin (145). Vanchrobactin is also a linear catechol but resembles enterobactin in that it contains serine linked to the catechol.

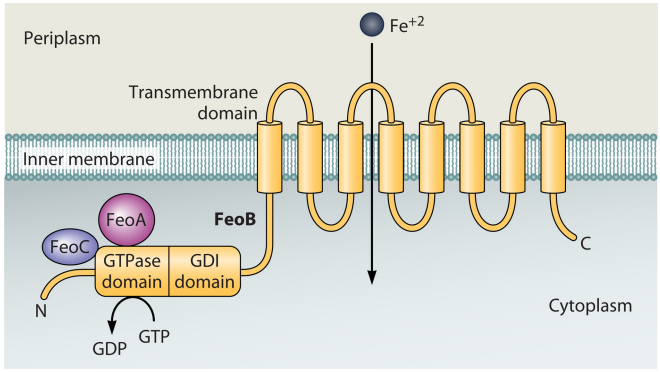

FIG 6 The ferrous iron transporter Feo. FeoB is localized to the cytoplasmic membrane. The accessory proteins FeoA and FeoC likely associate with the N-terminal cytoplasmic domain of FeoB. GDI, guanosine nucleotide dissociation inhibitor.

Transport of Unchelated Iron: Ferrous and Ferric Iron Transporters

Although siderophores have extremely high affinities for iron and can promote growth under many conditions of limited iron availability, they must not be sufficient for iron acquisition under all conditions, since all the vibrios tested have been found to have additional systems. This suggests that there has been selection for iron transport systems that can provide iron under conditions where the siderophores do not meet the cell's need for iron. In environments where only ferrous iron is available, for example, the ferric iron-binding siderophores would not efficiently transport iron. Transporters for both ferric iron and ferrous iron that is not complexed to a carrier have been identified in *Vibrio* spp.

FeoABC, a ferrous iron transporter, is found in all the *Vibrio* species analyzed (31, 146). This system is likely the most ancient bacterial iron transport system, since homologs of the *feo* genes are widely distributed among bacterial species, and prior to the evolution of oxygen in the Earth's atmosphere, Fe^{2+} would have been the predominant iron species. The Feo transporter was first described in *E. coli* (147) and consists of FeoB, an 85-kDa cytoplasmic membrane protein, and two small (~8.5-kDa) cytoplasmic proteins, FeoA and FeoC (Fig. 6). FeoB is the most highly conserved of these proteins in the vibrios, while FeoC is the least conserved (146). In *V. cholerae*, the *feoABC* genes form an operon, and all 3 genes are required for Feo-mediated iron transport (146, 148). Although this system has not been fully characterized, it is thought that FeoB forms the ferrous iron transport channel in the cytoplasmic membrane and that FeoA and FeoC serve accessory functions. FeoB is embedded in the cytoplasmic membrane and has a cytoplasmic, amino-terminal extension with homology to eukaryotic G proteins. The GTPase activity is required for transport, but its precise function has not been established (149). Similarly, the roles of FeoA and FeoC are poorly understood. Interactions between *V. cholerae* FeoB and FeoC have been shown (146). In *Salmonella*, FeoC has been shown to stabilize FeoB by providing

protection against protease degradation (150), and it may play a similar role in the *Vibrio* Feo transporter. FeoA has also been shown to interact with FeoB in *Salmonella* (151), but how FeoA functions in ferrous iron transport is unknown. Another question that remains to be answered is how ferrous iron gains access to the periplasm for transport by Feo. No ferrous iron transporter has been identified in the outer membrane. The ferrous iron may diffuse through porins or other relatively nonspecific channels, consistent with the lack of a requirement for TonB for Feo-mediated iron transport (152).

In *V. cholerae*, ferrous iron transport is facilitated by a cytoplasmic membrane protein designated VciB (152). The transport of ferrous iron and growth of bacteria are increased when VciB is present in addition to a ferrous iron transport system, including *V. cholerae* Feo, *E. coli* Feo, or *Shigella flexneri* Sit (152). The lack of specificity for *V. cholerae* Feo suggests that VciB does not recognize and interact with the ferrous iron transporter but may serve a more general function, such as in the reduction of ferric iron to ferrous iron for subsequent transport. Unlike most of the *V. cholerae* iron transporters, which have homologs in the other *Vibrio* species, VciB has few relatives in the genome sequence databases. VciB is found in all the sequenced *V. cholerae* genomes and may play a unique role in *V. cholerae* iron metabolism. Although it does not have homology to VciB, a ferric reductase has been identified in *V. vulnificus* (153), and it may play a similar role as VciB in making iron more available for transport via Feo.

V. cholerae also has a transporter for ferric iron, FbpABC, an ABC-type transporter in the cytoplasmic membrane (148). Based on homology with other ABC transporters, it was proposed that ferric iron bound to FbpA, the periplasmic binding protein component of the system, is delivered to FbpBC in the cytoplasmic membrane, which uses ATP hydrolysis to drive the transport of iron into the cytoplasm. As noted for the ferrous iron ligand for the Feo system, ferric iron transport by Fbp is TonB independent (148), and no specific outer membrane receptor has been identified. FbpABC is widely conserved in the *Vibrionaceae* and appears to represent a common mode of ferric iron acquisition in this family.

A third system for the transport of iron not complexed to a siderophore has been identified in *V. cholerae*. VctP, which is the periplasmic binding protein component of the Vct system, one of two *V. cholerae* transporters for catechol siderophores, also recognizes iron in the absence of the catechol. These two transport functions can be separated genetically. A tyrosine-to-phenylalanine substitution had no effect on ferri-catechol transport but eliminated the transport of iron in the absence of the siderophore (154). Thus, the Vct system can transport iron in either the presence or absence of the catechol siderophore.

IRON ACQUISITION WITHIN THE HOST

Vibrios are often found in association with other organisms. These associations present both opportunities for the bacteria to take advantage of new iron sources and hazards of outcompetition by host iron-binding proteins.

In humans, most of the iron is complexed to heme, primarily in red blood cell hemoglobin but also in heme proteins in other cells. Ferritin and Fe-S proteins account for much of the remainder of intracellular iron. Potential sources of iron in extracellular fluids are host iron-binding proteins, transferrin in the circulation and lactoferrin in secretions. However, these proteins are usually relatively unsaturated with iron, reducing the level of available iron in serum and secretions to a level below that needed to support microbial growth (155–159). Furthermore, lactoferrin has been found to have bactericidal activity against *V. cholerae* (160, 161). Lactoferrin can be cleaved by proteases to yield the antimicrobial peptide lactoferricin, which is active against a variety of Gram-negative pathogens (162), and it is possible that this accounts for the bactericidal effect against *V. cholerae*. In addition to the effects of iron-binding proteins, the vertebrate hormone hepcidin helps starve invading pathogens for iron by regulating the absorption of iron and its distribution within the body, reducing available iron in response to infection (158, 163, 164). This withholding of iron by host proteins and the active sequestration of iron in mammals in response to infection have been termed nutritional immunity (165, 166). Conditions that result in increased available iron in humans are associated with higher rates of infection and more severe infections. Hemochromatosis is associated with a variety of severe infections (167), including *V. vulnificus* (168) and non-O1 *V. cholerae* (169) infections. *V. vulnificus*, in particular, is associated with infections in people with iron overload (168, 170–172), and this can be reproduced in an animal model (173). It has been suggested that the sensitivity of hemochromatosis patients to fatal *V. vulnificus* infections is due, in part, to a failure to produce sufficient hepcidin (174). Studies using a hepcidin-deficient mouse model demonstrated that reduced hepcidin levels were associated with increased bacteremia, while administration of hepcidin agonists prevented death from *V. vulnificus* infection in these mice (175). The abrogation of this iron-withholding nutritional immunity favors growth of the pathogen.

Not surprisingly, many bacteria that infect humans or other vertebrates have evolved mechanisms to circumvent nutritional immunity and acquire iron bound to host proteins. These mechanisms include the expression of bacterial genes encoding iron acquisition systems in the host environment. Evidence that iron transport genes are expressed during infection has been obtained by using animal models or experimental infections of humans. Lombardo et al. (176) used *in vivo* expression technology (IVET) to determine the *V. cholerae* genes expressed during infection of human volunteers or in an infant mouse model. *fhuC*, encoding the ATPase of the hydroxamate transport system, was identified in both infected humans and the mouse model. Transcriptome sequencing (RNA-seq) analysis of *V. cholerae* isolated from the intestines of mice showed that a number of iron transport genes were highly induced in mouse (177). Furthermore, analysis of vibrios isolated directly from infected patients provided evidence for iron transport gene expression during naturally occurring infections. Genes for vulnibactin synthesis and the ferric vulnibactin receptor were detected in *V. vulnificus* obtained from the tissue of a patient with a severe soft tissue infection (178). Similarly, transcriptional profiling of *V. cholerae* obtained from stool of cholera patients showed the expression of the *vct* and *fbp* iron transport genes (179), and Fbp was also detected in the proteome of *V. cholerae* obtained from human stool (180). In contrast, neither *V. cholerae* (177) nor *V. parahaemolyticus* (181) showed significant expression of iron transport genes when isolated from the intestine of experimentally infected infant rabbits. This suggests that rabbit intestine is not as iron limited as human or mouse intestine and may not be the best model for studying iron transport *in vivo*.

Because multiple iron transport genes and proteins may be expressed in the host, it is not clear which of these systems are

required for iron acquisition and colonization. Several studies have used mutational analysis to assess the roles of specific iron transporters in acquiring iron from the host by infecting vibrios. The heme uptake gene cluster in *V. fischeri* contributes to symbiotic colonization of the squid light organ (182). The heme uptake genes were expressed during colonization of the squid light organ, and a mutant containing a deletion of the heme transport genes had reduced fitness in colonization. In contrast, heme transport was not essential for *V. cholerae* colonization in a mouse model of infection (120).

In the vertebrate host, transferrin is one of the iron-binding proteins that can limit bacterial growth. Some vibrios have the ability to acquire iron from transferrin, either directly or through their siderophores. Transferrin receptors, which bind host transferrin at the cell surface, where the iron is removed for transport, have been described for several groups of pathogens, including *Neisseria* spp. In the case of *Neisseria gonorrhoeae*, the transferrin receptor is required for human infection (183). Until recently, transferrin receptors had not been identified in *Vibrio* species. The lack of identification of transferrin-binding proteins on the surface of vibrios may be a result of the specificity of the receptors for host transferrin. A recent analysis of *V. vulnificus* biotype 2 strains, which cause disease in eels as well as humans, identified a receptor for eel, but not human, transferrin (184). This receptor, Vep20, has homology (~30% identity) to the *Neisseria* receptor for human transferrin, TbpA (185). Vep20 is required for full virulence and is plasmid encoded (184). Plasmids carrying the *vep20* gene were also found in other fish-pathogenic vibrios, including *V. harveyi*, suggesting horizontal transmission of this iron acquisition gene.

Among those vibrios that lack transferrin receptors, the secreted siderophore may be able to capture iron from transferrin. *V. vulnificus* type 2 (186), *V. parahaemolyticus* (187), and *V. damsela* (188) use their siderophores for removal of iron from human transferrin. Okujo et al. (189) showed that *V. vulnificus* produced an extracellular protease that cleaved transferrin and lactoferrin, making the iron more accessible to the bacteria. However, subsequent studies by Shin et al. (190) and Kim et al. (191) demonstrated that the metalloprotease VvpE was not required for iron uptake from holotransferrin; the siderophore vulnibactin allowed *V. vulnificus* to use transferrin iron irrespective of the presence of the protease.

While many siderophores are able to remove iron from transferrin *in vitro*, this does not ensure that they can acquire transferrin iron *in vivo*. In human, siderocalin (lipocalin 2) can bind catechol siderophores and remove them from the circulation (192–194). Not to be outwitted, bacteria have evolved siderophore modifications that defeat the antisiderophore properties of siderocalin. For example, some enteric pathogens glucosylate enterobactin, making it unable to be bound by siderocalin (43). It was originally reported that the *V. cholerae* siderophore vibriobactin was also a stealth siderophore, able to escape siderocalin binding because of its weaker negative charge and phenolate-oxazoline coordination mode (195). A subsequent study by Allred et al., however, showed that siderocalin shifts the coordination mode of vibriobactin from the phenolate-oxazoline mode to the distinct catecholate mode, allowing high-affinity binding by siderocalin (196).

Vibriobactin synthesis was not required for infection or fluid accumulation in an infant mouse model of infection (197). Similarly, a mutant defective in heme transport had no effect in this model (120), although a mutant defective in both the siderophore and heme iron uptake systems showed some reduction in colonization (198). A mutant defective in both the TonB1 and TonB2 systems also had reduced fitness in the infant mouse model (135), but testing of mutants defective in each of the known iron transport systems has not revealed any single system that is essential for *V. cholerae* colonization in the mouse model (148, 152, 198). Because *V. cholerae* has so many iron transport systems, there may be compensation for the loss of any one system. It is also likely that subtle losses in fitness cannot be detected in short-term animal experiments. In nature, even a small reduction in fitness due to the loss of an iron transporter may result in a loss of the mutant from the population. *V. cholerae* would not be expected to retain multiple iron transport systems if each one did not provide some advantage in a particular environment.

IRON AND REGULATION OF GENE EXPRESSION

Regulation of Iron Transport Systems

Although iron is essential, too much iron is lethal. Intracellular iron that is not bound to proteins or chelated can act as a catalyst for the Haber-Weiss reaction and participate in Fenton reactions, leading to the generation of toxic hydroxyl radicals (199, 200). Hydroxyl radicals damage DNA, unsaturated lipids, and proteins, resulting in increased mutations and cell damage or death (201, 202). Thus, any excess iron is sequestered in the cell, and the bacteria respond to excess iron by rapidly repressing iron uptake systems. There is a coordination of iron transport with the metabolic activities of the cells and tight regulation of iron transport to meet, but not exceed, the demand for iron.

The major regulator of iron transport in Gram-negative bacteria is Fur. Fur is an iron-binding transcriptional regulator that was first identified in *E. coli* (200). It forms a homodimer, and in the presence of iron, the iron-Fur complex binds to specific sites on the DNA. These sites, termed Fur boxes, are typically located within the −10 and −35 regions of the promoters of iron-regulated genes, and the association of Fur with sites at this location blocks transcription.

All *Vibrio* spp. analyzed thus far have a Fur homolog. *V. cholerae fur* was identified by Litwin et al. (203), and a *fur* mutant was found to derepress the expression of two genes involved in iron transport, *irgA* and *viuA*, suggesting that it functioned much like Fur in *E. coli* (204). Crystallization of the *V. cholerae* Fur protein provided evidence for two metal-binding sites per monomer: a regulatory site where iron or other divalent metal cations can bind and an auxiliary zinc-binding site (205). No binding to DNA occurred when the metals were removed from the protein. There was no evidence that metal binding required any of the conserved cysteines in the protein (205). The Fur protein is relatively abundant for a transcriptional regulator; there are ~2,500 copies per cell in exponentially growing *V. cholerae* bacteria, increasing to ~7,500 copies in stationary-phase cells (206). Although Fur is relatively abundant, its levels are modulated in response to iron and the metabolic status of the cell. There is moderate negative regulation of *fur* by Fe-Fur in *E. coli* (207), while studies of *V. vulnificus* have shown positive regulation of *fur* by Fur in the absence of iron (208, 209). These types of autoregulation may control the ratios of Fur to Fe-Fur in the cell to limit the formation of aberrantly active dimers between iron-bound and iron-free Fur.

fur is also positively regulated by cyclic AMP receptor protein (Crp) (207), and this may help couple cell metabolism to iron uptake.

Transcriptional analysis of Fur regulation in *V. cholerae* revealed positive as well as negative effects on transcription, although regulation was predominantly negative (210). Fur repressed the genes involved in iron acquisition when the cells were grown under iron-replete conditions but positively affected the expression of *ompT*, encoding a major porin protein (210, 211). For most of the negatively regulated genes, a putative Fur box is found in the −10 and −35 regions, but the binding site for Fur in the *ompT* promoter is upstream, and increased expression may be due to Fur competing with a negative regulator at this binding site.

A more recent analysis of transcriptional regulation of Fur discriminated between direct and indirect effects. Davies et al. (212) used chromatin immunoprecipitation sequencing (ChIP-seq) to identify the Fur-binding sites in the *V. cholerae* genome. This study verified a number of the sites predicted by genetic and transcriptional analyses and revealed new sites, including several small RNAs (sRNAs). Based on their analysis, a revised 21-bp Fur box consensus sequence was defined, and the site of the Fur box relative to the transcriptional start site was found to be variable.

There is also evidence for strain differences in the regulation of iron transporters. There are two distinct biotypes of *V. cholerae*, classical and El Tor, which differ in virulence, environmental persistence, hemolysin production, and other phenotypes. The El Tor biotype was found to have higher expression levels of iron transport genes than classical *V. cholerae* (213), and El Tor and non-O1 strains were noted to produce larger amounts of vibriobactin and to have greater resistance to chemical iron chelators than classical strains (5). Whether this is related to differences in Fur or other regulation is unknown.

Fur has been shown to play similar roles in *V. vulnificus* (214–216), *V. anguillarum* (217, 218), and *V. salmonicida* (219). In each of these vibrios, Fur negatively regulates the expression of genes for iron acquisition systems, thereby reducing the chance of toxicity due to iron overload.

Many of the vibrios secrete hemolysins that may indirectly aid in iron acquisition by lysing erythrocytes or other host cells, releasing internal heme and iron. Fur negatively regulates the expression of hemolysins in *V. cholerae* (220), *V. parahaemolyticus* (221), and *V. vulnificus* (215). *V. vulnificus* produces a protease that aids in the acquisition of heme from heme-albumin (222). This proteolytic activity is not regulated by iron but is induced by heme or hemoglobin.

Although Fur acts as a global regulator to repress iron transport systems, there are a number of examples of additional regulators that affect individual iron transport systems. In *V. anguillarum*, anguibactin transport is positively regulated by AngR and Taf (223–225). Taf is a transcriptional activator (226), while AngR is a bifunctional protein required for the synthesis of anguibactin as well as for the regulation of its transport proteins (225, 227). Other examples of positive regulation are the activation of *irgA*, encoding a *V. cholerae* enterobactin receptor, and *hupR*, encoding the *V. vulnificus* heme receptor, both of which are divergently transcribed from positive regulators. IrgB activates the transcription of *irgA* (228, 229), and HupR induces *hupA* (230). Both IrgB and HupR are members of the LysR family of positive transcriptional activators (228, 230). A number of other *Vibrio* iron transport genes, including *vctA*, have known or putative regulators that are divergently transcribed. In each of the systems with positive regulation, the genes for the activators, as well as the iron transporters, are negatively regulated by Fur. This arrangement amplifies the response to low-iron environments and allows a much greater fold change in expression as a function of the iron concentration than would Fur regulation alone.

Iron and Regulatory RNAs

Transcriptional analyses of iron and Fur regulation have shown that the concentration of iron and the presence or absence of Fur have widespread effects on the cell. Not only is iron transport tightly controlled by Fur, but changes in superoxide dismutase (SodB), TCA cycle enzymes, biofilm formation, and a variety of other phenotypes are influenced by Fur. While the effects on iron transport genes are direct, many of the other genes are indirectly regulated by Fur via RyhB, an sRNA. RyhB, first described in *E. coli*, repressed the synthesis of the iron-containing superoxide dismutase, encoded by *sodB*, and aconitase, encoded by *acnB*, among others, by controlling mRNA stability and translation (231, 232). RyhB can bind to the transcripts of these genes in the presence of Hfq and direct their degradation by RNase E (233). The expression of *ryhB* is negatively regulated by Fur (232). Thus, Fur can positively affect gene expression by reducing the levels of the repressor RyhB. RyhB provides a mechanism for linking cellular metabolism to iron availability and shutting down iron-greedy enzymes and iron storage proteins when iron is less plentiful (234). The complexity of the regulation and level of control of Fur, RyhB, and their targets is further illustrated by the fact that RyhB influences the levels of Fur. RyhB downregulates the expression of Fur in *E. coli* and is predicted to regulate *fur* in *Vibrio* spp. (235). There is complementarity between RyhB and the translation initiation region of the *V. cholerae fur* gene that may result in the downregulation of *fur* mRNA.

In *V. cholerae*, RyhB plays a role similar to that of the *E. coli* homolog, linking iron acquisition and overall metabolism. Analysis of the RyhB regulon in *V. cholerae* showed that RyhB influenced the synthesis of superoxide dismutase, TCA cycle enzymes, and proteins containing heme or iron-sulfur clusters (21, 236). However, *V. cholerae* RyhB is considerably larger than its *E. coli* homolog (>200 nucleotides, compared to 90 for *E. coli*) and has a larger regulon, influencing the expression of genes involved in motility, chemotaxis, and biofilm formation. A mutant with a deletion in *ryhB* had reduced chemotactic motility and was unable to form wild-type biofilms in low-iron medium (21). The addition of iron restored biofilm formation in the *V. cholerae ryhB* mutant, but excess iron did not enhance biofilm formation in the wild-type strain (21). This suggests that the *ryhB* mutant is iron stressed in biofilms, and the inability of the *ryhB* mutant to properly maintain iron homeostasis likely contributes to the defects in motility and biofilm formation.

The role of RyhB in iron regulation is even more complex in *V. parahaemolyticus*, where RyhB positively regulates the production of the siderophore vibrioferrin (237). RyhB, with the aid of Hfq, can directly bind to and stabilize the 5′ untranslated region (UTR) of *pvsO*, a polycistronic message for vibrioferrin biosynthesis, leading to increased vibrioferrin synthesis.

In *V. anguillarum*, another RNA is involved in the regulation of siderophore transport. An antisense RNA, RNAα, specifically controls the expression of the transport genes *fatA* and *fatB* by binding to the *fatA* and *fatB* mRNAs and reducing their transla-

TABLE 2 Regulation of iron transport by signals in addition to iron availability[a]

Environment	Most likely iron source(s)[b]	Environmental signals[c]	Possible *Vibrio* regulator(s)	Effect(s) on *Vibrio* iron transporters
Marine surface waters	Fe^{3+} compounds	O_2 present Neutral or alkaline pH	Fnr,[d] ArcAB[d] PepA[e]	↑ siderophore ↓ Feo
Sediments, solid surfaces, or biofilms	Fe^{2+} compounds	Low O_2 concn, reduced diffusion, and crowding in biofilm	Fnr,[d] ArcAB[d] LitR,[f] quorum sensing[f]	↑ Feo ↓ siderophore, ↓ heme transporters
Host	Heme and transferrin in blood and tissues, lactoferrin in secretions, and Fe^{2+} iron complexes in intestine	Temperature Specific C sources Specific N sources in squid Low O_2 concn in intestine Bile or antimicrobial peptides in intestine	H-NS[d] Crp[f] GlnD[f] Fnr,[d] ArcAB[d] Cpx[f]	↑ heme transporters ↑ TonB ↑ siderophore ↓ siderophore, ↑ Feo ↑ heme transporters, ↑ siderophore

[a] Shown is a summary of the effects of environmental conditions on the regulation of iron transport systems. Conditions that have been shown to affect specific iron transporters are discussed in the text. Arrows indicate a positive (up arrow) or negative (down arrow) effect on expression of the iron transport genes.
[b] Iron sources present in each environment are predicted based on the known distributions of iron complexes.
[c] Environmental signals are predicted based on conditions known to exist in each environment.
[d] Regulator that affects iron transport in other species and for which homologs are present in *Vibrio* spp.
[e] Regulator that is present in *Vibrio* spp. and is known to regulate genes in response to the stated conditions but that has not been shown to regulate iron acquisition genes.
[f] Regulator that is known to affect *Vibrio* iron transport.

tion (238, 239). Fur is required for RNAα synthesis and controls transcription initiation, independent of the iron status of the cell. It is not known how Fur regulates gene expression in the absence of iron. However, iron-independent Fur regulation of gene expression was noted in an analysis of the Fur and iron regulons in *V. cholerae* (210). Iron plays a role in RNAα expression, but the effect of iron is posttranscriptional: RNAα is stabilized in cells grown in the presence of iron (238). The effect of RNAα in addition to Fur allows tighter repression of FatA and FatB synthesis in the presence of iron.

Other Environmental Sensors and Regulators for Iron Acquisition

In addition to iron, there are a variety of other environmental factors that influence the expression of iron transport genes, including temperature, oxygen, carbon sources, and quorum-sensing molecules. These factors, summarized in Table 2, may correlate with the form of iron available and serve as indirect signals for specific iron transport systems. Higher oxygen levels are associated with the presence of ferric, rather than ferrous, iron. Thus, the presence of oxygen may signal that it would be advantageous to express ferric iron transporters, while anoxic environments may increase the synthesis of ferrous iron transporters (240). Similarly, higher temperatures signal a mammalian host environment and could favor the expression of transporters that acquire iron from host proteins. Expression of the cell's entire repertoire of iron transporters under all conditions would be metabolically costly and could lead to the import of toxic levels of iron. Thus, additional layers of regulation would allow the cell to optimize iron acquisition and growth by tuning the expression of the transport genes to the most likely available iron source.

Regulation in response to signals found within the host. The sources of iron available to vibrios in the host vary depending on whether the bacteria are found on body surfaces or in tissues or blood. Heme is the most abundant iron source in animal hosts, and linking the expression of heme or hemoglobin receptors to host conditions allows the bacteria to take advantage of this iron source. In *V. vulnificus*, the heme receptor gene *hupA* is regulated by temperature: there is a significant increase in the level of transcription at 40°C compared to that at 30°C (241). HupA is required for maximal virulence in mice, and linking its expression to higher temperatures allows its expression in the mammalian host but not in the generally cooler, external environments. In contrast, *V. salmonicida*, which causes cold-water vibriosis in Atlantic salmon, produces siderophores only at lower temperatures (242). Siderophores and iron-regulated outer membrane transport proteins are synthesized at 10°C, the water temperature at which hemorrhagic disease occurs, but not at temperatures of 15°C or higher. The mechanism by which temperature affects the expression of *Vibrio* iron transport genes has not been determined, but the nucleoid-associated protein H-NS is a possible mediator. H-NS is found in *Vibrio* and is known to regulate gene expression as a function of temperature in other species.

Other signals in the host include the presence of specific carbon and nitrogen sources; however, these and other signals, such as O_2 concentration and pH, are not limited to the host environment but may be found in other habitats. It is the ability of the vibrios to integrate multiple environmental signals to regulate iron transport that allows them to more precisely determine their location and possible iron sources.

Effects of metabolism on expression of iron transport genes. Carbon metabolism influences the expression of *Vibrio* iron transport genes. Crp binds upstream of the *V. vulnificus hupA* gene and activates its expression (241). Crp also induces the expression of *tonB3* (137) and the aerobactin receptor gene *iutA* (243) in *V. vulnificus*. Crp and cAMP may couple growth on carbon sources other than glucose with the increased need for iron required for full metabolism of these substrates. While glycolysis does not require iron-containing enzymes, other pathways, such as the Entner-Doudoroff pathway and the TCA cycle, have enzymes that require iron for activity. Under low-iron conditions, *E. coli* shifts

its metabolism to increase glycolysis and decrease the level of TCA cycle enzymes (244). It is likely that vibrios alter their metabolism in a similar way. In the absence of glucose, additional iron would be needed for carbon metabolism via pathways other than or in addition to glycolysis.

Another example of a link between metabolism and iron transport has been identified in *V. fischeri*, which couples nitrogen metabolism to iron acquisition. A mutation in *glnD*, which acts as a nitrogen sensor, not only caused a defect in nitrogen metabolism but also reduced siderophore synthesis by these bacteria (38). The *glnD* mutant was unable to persist in the squid light organ, but excess iron restored persistence to wild-type levels, indicating that the colonization defect was a result of the effect of the *glnD* mutation on iron acquisition. Thus, the presence of specific nitrogen sources in the squid light organ may serve as a signal for this host environment and increase the synthesis of the siderophore needed for colonization.

Quorum sensing regulation and iron transport. Several studies have also linked quorum sensing to iron metabolism in *Vibrio* spp. Quorum sensing modulates a variety of vibrio activities, including biofilm formation and the expression of virulence factors. In *V. vulnificus*, quorum sensing repressed *vvsA* and *vvsB*, which encode a NRPS required for vulnibactin synthesis. The primary quorum sensing regulator, SmcR, binds upstream of the *vvsAB* transcription start site and overlaps the Fur-binding site. Under high-iron conditions, Fur repressed *vvsA* transcription, while in low-iron environments, SmcR regulated the expression of the siderophore biosynthesis genes (245). Using a *luxS* mutant defective in the synthesis of autoinducer 2 (AI-2), Kim and Shin observed an increase in the synthesis of the siderophore receptors for aerobactin (IutA) and heme (HupA), suggesting that they are also repressed by quorum sensing (246). Additional studies revealed a more complex connection between iron and quorum sensing (247). Fur bound at two sites upstream of the transcriptional start site of *smcR*, repressing the production of SmcR under high-iron conditions. However, the affinity of Fur at these sites was low enough that *smcR* was induced by quorum sensing at a high cell density, even under iron-rich conditions. In low-iron environments, Fur did not bind, and *smcR* expression was regulated solely by quorum sensing. This dual control by iron (via Fur) and quorum sensing (via SmcR) allows fine-tuning of iron acquisition. At a high cell density, higher levels of the siderophore in the environment, representing a common good coupled with the reduced need for iron as the cells enter stationary phase, could result in cells importing too much iron. Monitoring cell density as a secondary level of negative regulation provides tighter control over cellular iron levels. This cross talk between iron and quorum sensing regulation may be a common theme in vibrios: Septer et al. (248) showed that Fur represses LitR, a positive regulator of *V. fischeri* quorum sensing that is a homolog of *V. vulnificus* SmcR.

Membrane stress response and regulation of iron transport. Another environmental sensor that plays a role in the regulation of *Vibrio* iron transport is the two-component Cpx pathway that responds to envelope stress (249). Iron starvation in *V. cholerae* led to Cpx activation, and overexpression of the response regulator gene *cpxR* induced the expression of a number of iron-regulated genes, including those for iron transport proteins. The Cpx response was inhibited by the addition of iron, suggesting that iron is a common component of Cpx-inducing signals in *V. cholerae* (249). Cpx may respond to the accumulation of potentially toxic apo-cofactors for iron-containing proteins in the membrane or periplasm, or it may be a response to the large number of membrane proteins induced by iron starvation. Additionally, the presence of bile and membrane-damaging antimicrobial peptides in the intestine could activate the Cpx response and signal the need for increased levels of iron transporters in the host environment. Thus, Cpx may aid in the response to membrane perturbations associated with iron stress and help coordinate cellular functions in response to iron starvation.

Growth on surfaces induces iron transport genes. In *V. parahaemolyticus*, some iron transport proteins were induced by growth on a surface (250). A comparison of genes expressed in cells grown on solid medium to those expressed in cells grown in broth showed the induction of a large number of genes, including those encoding swarming motility proteins, virulence factors, and sensory enzymes involved in chemoreception and cyclic di-GMP (c-di-GMP) signaling. Iron-regulated genes, including those encoding the enterobactin and ferrichrome receptors, were also induced when the bacteria were grown on the surface of agar medium. This finding is consistent with data from previous studies showing that restricted diffusion of iron in agar media limits its availability to *V. parahaemolyticus* cells growing on surfaces, and iron is a key signal for swarmer cell differentiation (251). Differentiation in response to iron limitation requires Fur, but it is not known whether the effect of Fur is direct or indirect (L. McCarter, personal communication).

Iron Regulates Expression of Genes for Systems Other than Iron Metabolism

In addition to its role as an essential element in maintaining cell function, iron also serves as an environmental sensor for a wide range of bacterial activities. In particular, it has long been noted that environments deficient in iron trigger the synthesis of virulence factors such as diphtheria toxin (252) and Shiga toxin (253), suggesting that low iron levels are a signal that bacteria use to sense that they are within a vertebrate host. This iron regulation is dependent on Fur or Fur-like repressors (254–256), representing another example of virulence factors coopting basic cellular regulators of metabolism for control of virulence gene expression. In *V. cholerae*, Fur positively regulates genes located in the *V. cholerae* pathogenicity island, which encodes the toxin-coregulated pilus (TCP), a major virulence factor of *V. cholerae*, as well as genes within the mega-integron (210). Unlike the effects of Fur on iron transport systems, the regulation of pathogenicity island genes was independent of the iron concentration. Consistent with the regulation of TCP synthesis or assembly, a *fur* mutant exhibited very weak autoagglutination, independent of the iron concentration. Furthermore, the *fur* mutant had a strong phenotype in the infant mouse model of cholera. The *fur* mutant was highly attenuated and failed to compete effectively with the wild type for colonization of the mouse intestine (210).

CONCLUSIONS

Iron acquisition is critical to the survival of *Vibrio* spp. These bacteria inhabit a wide variety of habitats and encounter iron in different forms depending on the habitat. The available iron may be ferric or ferrous and may be in insoluble hydroxides or part of heme or iron sulfur clusters in host proteins. *Vibrio* species have evolved systems to acquire iron in all of these forms, and there is evidence that iron transport systems may have been acquired from

other bacterial species by horizontal transmission. Thus, these bacteria are well adapted for survival in marine habitats and for rapid adaptation to new environments when they transition to a host or to polymicrobial communities. The selective pressures that shape the repertoire of systems found in any of the *Vibrio* spp. may be positive (ability to acquire iron in a new environment or to reduce competition with other bacteria) and negative (TonB-dependent receptors may be antigenic or may be receptors for bacteriophages). From the vibrio point of view, it is apparent that more is better when it comes to iron transport systems.

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