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Iron acquisition in *Vibrio cholerae*

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Abstract *Vibrio cholerae*, the causative agent of cholera, has an absolute requirement for iron and must obtain this element in the human host as well as in its varied environmental niches. It has multiple systems for iron acquisition, including the TonB-dependent transport of heme, the endogenous siderophore vibriobactin and several siderophores that are produced by other microorganisms. There is also a Feo system for the transport of ferrous iron and an ABC transporter, Fbp, which transports ferric iron. There appears to be at least one additional high affinity iron transport system that has not yet been identified. In iron replete conditions, iron acquisition genes are repressed by Fur. Fur also represses the synthesis of a small, regulatory RNA, RyhB, which negatively regulates genes for iron-containing proteins involved in the tricarboxylic acid cycle and respiration as well as genes for motility and chemotaxis. The redundancy in iron transport systems has made it more difficult to determine the role of individual systems in vivo and in vitro, but it may reflect the overall importance of iron in the growth and survival of *V. cholerae*.

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Introduction

Vibrio cholerae causes the severe diarrheal disease cholera. During infection of the human host, *V. cholerae* colonizes the small intestine by adhering to the epithelial cells through the action of the toxin-coregulated pilus (TCP). *V. cholerae* secretes a potent enterotoxin (CT) that deregulates cAMP production in the epithelial cells, resulting in massive ion and water efflux (Kaper et al., 1995). Cholera is still a significant public health threat in areas where clean water is not widely available (Sack et al., 2006). Outside of the human host, *V. cholerae* is an aquatic organism, where it can exist in a free planktonic form, or in association with fish, crustaceans or cyanobacteria (Faruque et al., 1998; Reidl and Klose, 2002; Reen et al., 2006). *V. cholerae* has multiple iron transport systems, that may allow optimal acquisition of this essential element in each of these environments. The *V. cholerae* genome as been completely sequenced (Heidelberg et al., 2000), and iron transport systems are encoded on both of its two chromosomes (Fig. 1).

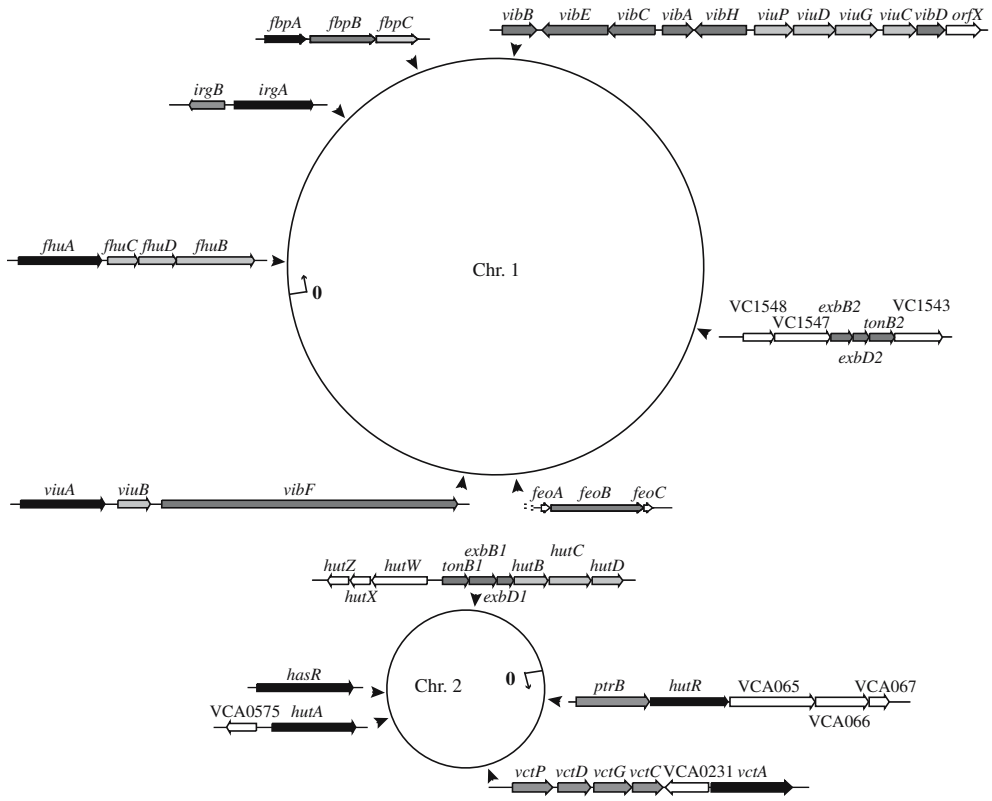

Fig. 1 Genetic maps of *V. cholerae* iron acquisition genes. The approximate locations of the genetic regions are indicated on the circles representing chromosomes 1 and 2. The direction of transcription is indicated by the arrows. The lines with arrows inside the circles represents nucleotide 0 and the direction of nucleotide numbering in the TIGR database, and TIGR locus designations are used to identify unnamed genes Heidelberg et al. (2000)

Siderophores

Vibriobactin biosynthesis

Vibrio cholerae synthesizes the catechol siderophore vibriobactin, which consists a norspermidine backbone the three dihydroxybenzoate (DHB) moieties (Fig. 2). One of the dihydroxybenzoate groups is attached directly to the norspermidine, while the other two are attached through cyclized threonine bridges (Griffiths et al., 1984). The pathway for the synthesis of dihydroxybenzoate from chorismate is the same as for enterobactin and requires VibABC (Wyckoff et al., 1997). Vibriobactin is assembled from DHB, threonine and norspermidine by an unusual nonribosomal peptide synthesis-type mechanism that has been

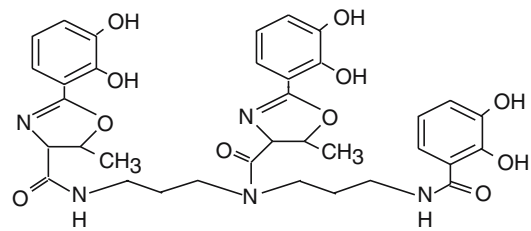

Fig. 2 The structure of vibriobactin Griffiths et al. (1984)

determined in the laboratory of Dr. Christopher T. Walsh and requires VibBDEFH (Keating et al., 2000; Crosa and Walsh, 2002; Di Lorenzo et al., 2004). Strains carrying mutations in any of the vibriobactin biosynthetic genes do not make a detectable siderophore, indicating that the vibriobactin is likely the sole siderophore produced by

V. cholerae (Wyckoff et al., 1997; Butterton et al., 2000; Wyckoff et al., 2001).

The vibriobactin genes map in two gene clusters that are separated by about 1.5 Mbp on the large chromosome, and each cluster contains genes for both vibriobactin biosynthesis and transport (Fig. 1). Genes for the synthesis and transport of a siderophore are generally grouped together on the bacterial chromosome, and it is unusual to have the genes for a single siderophore separated by a large distance. It is not known whether the two clusters were acquired separately or whether all of the vibriobactin genes were previously in a single cluster that was disrupted by a genomic rearrangement.

Siderophore transport and utilization

The *V. cholerae* iron transport genes are listed in Table 1. The vibriobactin utilization gene, *viuA*, encodes the outer membrane receptor for vibriobactin (Butterton et al., 1992; Litwin and Calderwood, 1994; Occhino et al., 1998). Downstream of *viuA* lies *viuB* (Fig. 1), and both genes are required for vibriobactin utilization. Plasmid-encoded *viuB* complemented an *Escherichia coli fes* mutation, leading to the model that ViuB is required for removal of iron from ferri-vibriobactin following its transport into the cytoplasm (Butterton and Calderwood, 1994).

Vibrio cholerae can use several siderophores that it does not itself make. Two enterobactin receptor genes are present in the *V. cholerae* genome (Mey et al., 2002). One of these, *irgA* (iron-regulated gene A), encodes a protein with sequence similarity to *E. coli* CirA and to a lesser extent with FepA (Goldberg et al., 1990b, 1992). The other receptor, VctA (*Vibrio* catechol transport gene A), has similarity to the enterobactin receptor FetA of *Neisseria gonorrhoeae* (Beucher and Sparling, 1995; Carson et al., 1999). Strains carrying mutations either *irgA* or *vctA* transported enterobactin normally, but the *irgA*, *vctA* double mutant was completely defective in enterobactin transport (Mey et al., 2002). The source of enterobactin used in these experiments was an unpurified siderophore secreted by *E. coli* K12, which is a mixture that includes standard cyclic enterobactin, as well as linear monomers, dimers and trimers of dihydroxybenzoyl serine (DBS), $(DBS)_2$ and $(DBS)_3$. It is not known which product, or combination of these products, is transported by IrgA and VctA.

The significance of enterobactin utilization for the growth and survival of *V. cholerae* is unknown. Enterobactin is not expected to be available to *V. cholerae* in significant quantities in vivo, since enterobactin is primarily secreted by bacteria in the colon, while *V. cholerae* colonizes the small intestine. Since *V. cholerae* could encounter enterobactin-producing organisms in sewage-contaminated waters, use of enterobactin may be more important during growth in the environment.

Vibrio cholerae also uses agrobactin (Ong et al., 1979; Griffiths et al., 1984) and a *Vibrio fluvialis*-derived siderophore that is probably fluvibactin (Andrus et al., 1983; Yamamoto et al., 1993). Fluvibactin and agrobactin have structures similar to that of vibriobactin, with norspermidine and spermidine backbones, respectively. A mutant defective in vibriobactin transport also failed to transport agrobactin, suggesting that ViuA transports agrobactin (Griffiths et al., 1984) and, given the similarity in structure, probably also fluvibactin. Enterobactin is also a catechol siderophore, but it is not transported by ViuA (Mey et al., 2002), and neither IrgA nor VctA transports vibriobactin (Wyckoff et al., 1999). Thus, although these receptors may transport several closely related catechol siderophores, they do not transport all catechol siderophores.

Vibrio cholerae has two periplasmic binding protein-dependent ABC transport systems for the transport of catechol siderophores. *viuPDGC* was identified within one of the vibriobactin biosynthetic gene clusters, while *vctPDGC* is linked to the enterobactin receptor *vctA* (Fig. 1). Mutants in either the *vctPDGC* or *viuPDGC* genes used both vibriobactin and enterobactin, but a *viuPDGC*, *vctPDGC* double mutant transported neither siderophore (Wyckoff et al., 1999; Mey et al., 2002). Thus, unlike the outer membrane receptors, ViuPDGC and VctPDGC can transport both vibriobactin and enterobactin.

As is common in enteric bacteria, *V. cholerae* uses ferrichrome as a source of iron (Griffiths

Table 1 Iron transport systems of *V. cholerae*

Genes	ORFs[b]	Ligand	Regulation by[a]	
			Iron	Fur
TonB-dependent receptors				
fhuA[c]	VC0200	Ferrichrome	Yes	Yes
viuA[d]	VC2211	Vibriobactin	Yes	Yes
irgA[e]	VC0475	Enterobactin	Yes	Yes
vctA[f]	VCA0232	Enterobactin	Yes	Yes
hutA[g]	VCA0576	Heme	Yes	Yes
hutR[h]	VCA0064	Heme	Yes	Yes
hasR[h]	VCA0625	Heme	No	No
Unnamed	VC0284	Unknown	Yes	Yes
btuB	VC0156	Putative vitamin B12	No	No
Periplasmic and cytoplasmic memebrane permeases				
fhuBCD[c]	VC0201-3	Ferrichrome	Yes[i]	Yes[i]
viuAPDGC[j]	VC0776-9	Vibriobactin and Enterobactin	Yes	Yes
vctPDGC[f]	VCA0227-30	Vibriobactin and Enterobactin	Yes	Yes
hutBCD[k]	VCA0914-6	Heme	Yes	Yes
feoABC[l]	VC2076-8	Ferrous iron	Yes	Yes
fbpABC[l]	VC0608-10	Ferric iron	Yes[m]	Yes[m]

[a] As determined in Mey et al. (2005b) and in references cited for individual genes

[b] ORF numbering is according to the TIGR database Heidelberg et al. (2000)

[c] Rogers et al. (2000)

[d] Butterton et al. (1992)

[e] Goldberg et al. (1990a), Goldberg et al. (1992) and Mey et al. (2002)

[f] Mey et al. (2002)

[g] Henderson and Payne (1993) and Henderson and Payne (1994b)

[h] Mey and Payne (2001)

[i] Only *fhuC* (VC0201) was significantly regulated in microarrays Mey et al. (2005b)

[j] Wyckoff et al. (1999)

[k] Occhino et al. (1998)

[l] Wyckoff et al. (2006)

[m] Only *fbpA* (VC0608) was significantly regulated in microarrays Mey et al. (2005b)

et al., 1984). The ferrichrome receptor gene *fhuA*, as well as the genes for transport of ferrichrome across the inner membrane, *fhuBCD* have been identified (Rogers et al., 2000) (Fig 1).

Heme

Vibrio cholerae uses both heme and hemoglobin as a source of iron (Stoebner and Payne, 1988). A heme receptor gene, *hutA*, was originally isolated from a cosmid clone that conferred the ability to use heme on an *E. coli* K12 strain carrying a separate plasmid encoding the *V. cholerae tonB1* system genes, as well as genes for transport of heme across the inner membrane (Henderson and Payne, 1993, 1994b; Occhino et al., 1998). A *V. cholerae hutA* mutant had only a modest reduction in heme utilization, though hemoglobin utilization was more dramatically reduced (Mey and Payne, 2001). When the *V. cholerae* genome sequence became available, a second gene, *hutR*, with similarity to *hutA* was identified. The *hutA*, *hutR* mutant could not use hemoglobin as an iron source and used heme very weakly (Mey and Payne, 2001). A third heme receptor gene was named *hasR* because of its similarity to the *hasR* hemophore receptor genes of *Pseudomonas aeruginosa* (Ochsner et al., 2000) and *Serratia marcescens* (Ghigo et al., 1997). However, no gene with similarity to known hemophores has been identified in the *V. cholerae* genome. Utilization

of heme was completely abolished in the *hutA*, *hutR*, *hasR* triple mutant, indicating that this is a complete list of the heme transporters (Mey and Payne, 2001).

While the transport of heme into gram negative bacteria has been widely studied, less is known about the fate of heme after it enters the cells. Adding heme to the medium suppresses the growth defect of a *V. cholerae* heme biosynthetic mutant, indicating that transported heme can be incorporated into cytochromes and possibly other heme-binding proteins (Henderson, 1993). We have identified an apparent cytoplasmic protein, HutZ, that is required for efficient heme utilization. *hutZ* is located in the *hutWXZ* operon that is divergently transcribed from *tonB1* system genes (Fig. 1). Only *hutZ* was required to complement the heme utilization defect of a polar mutation in *hutW*, and no function has been identified for *hutWX*. HutZ did not protect cells from heme toxicity; rather it appears to promote the use of heme as an iron source.

Strains of *Corynebacterium diphtheriae* or *Corynebacterium ulcerans* carrying a mutation in the heme oxygenase gene, *hmuO*, cannot use heme as an iron source, and this defect was completely suppressed by a plasmid containing the *V. cholerae hutWXZ* operon. HutZ was required for this effect, suggesting that HutZ could be a heme oxygenase. However, biochemical analysis of purified HutZ indicated that, although it bound heme, it did not specifically catalyze heme degradation (Wyckoff et al., 2004). The solution to this paradox has not been resolved. It is possible that HutZ catalyzes removal of iron from heme by a mechanism that was not detected in the heme oxygenase assay. For example, HutZ may require proteins or a cofactor not supplied in the assay. Alternatively, HutZ may have a function that promotes the efficient use of heme as an iron source without catalyzing heme degradation. As a soluble heme binding protein, HutZ could potentially carry heme from the heme transporters in the cell membrane to heme-containing proteins located throughout the cell. This is supported by the observation that heme bound to purified HutZ was efficiently transferred to heme oxygenase in vitro (Wyckoff et al., 2004). The mystery of HutZ function has only been made deeper by the recent solution of the crystal structure of the HutZ homologue alr5027 from *Nostoc* sp. Pcc 7120 (≤http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pl?pdbcode=1vl7≥). Alr5027 crystallized as a dimer with a fold characteristic of the pyridoxamine-phosphate oxidase family of FMN binding proteins.

TonB

Vibrio cholerae was the first organism in which two *tonB* systems were characterized (Occhino et al., 1998). The *tonB1* system genes are co-transcribed with the genes for the transport of heme across the inner membrane in an operon divergently transcribed from *hutWXZ* (Fig. 1) (Occhino et al., 1998; Wyckoff et al., 2004). The *tonB2* system genes are located in an operon with three ORFs of unknown function, one of which (VC1547) has sequence similarity with *exbB*. The *tonB2* system genes complement an *E. coli tonB* mutant, whereas the *tonB1* system genes do not.

The two TonB systems have both redundant and specific functions. Both TonB systems functioned well for the transport of vibriobactin, while the TonB2 system was required for the use of enterobactin and for heme transport via HasR. The heme receptors HutA and HutR functioned with either TonB1 or TonB2, but appeared to transport heme most efficiently when TonB1 is present (Mey and Payne, 2001; Seliger et al., 2001). Some of the amino acids responsible for the specificity of these interactions have been mapped. Changing TonB1 proline 237 to threonine, arginine or alanine allowed TonB1 to interact functionally with the *V. cholerae* enterobactin receptors. Receptors that function with TonB1 have a large hydrophobic amino acid immediately preceding the TonB box. Introducing a tyrosine or leucine in place of the aspartic acid preceding the TonB box in the enterobactin receptor IrgA allowed it to function with TonB1, although these altered IrgA proteins retained the ability to function with TonB2 (Mey and Payne, 2003).

The TonB1 protein is longer than either TonB2 or *E. coli* TonB and is expected to span the periplasm more easily than its shorter

homologues. Under high osmotic conditions, which would be expected to increase the volume of the periplasm (Stock et al., 1977), the efficient use of heme by *V. cholerae* was dependent upon the TonB1 system. A deletion in the proline-rich periplasmic-spanning domain of TonB1, reducing its size to that of TonB2, eliminated its ability to energize heme transport in conditions of high osmolarity, such as that found in seawater (Seliger et al., 2001). Interestingly, this effect was specific to heme, since the activity of the ferrichrome receptor FhuA was not affected by osmolarity, even when energized by TonB2 or the shortened TonB1. This raises the possibility that the heme receptors HutA and HutR might not extend as far into the periplasm as FhuA and may thus require a longer TonB, or they may interact with TonB in a way that is more sensitive to these changes in TonB1 (Seliger et al., 2001).

TonB-independent iron transporters

Feo

Feo is a poorly understood transporter of ferrous iron (Hantke, 1987; Kammler et al., 1993; Hantke, 2004; Cartrön et al., 2006). In general, Feo systems consist of at least two proteins. In *E. coli*, FeoA is a small protein of unknown function, while FeoB is a larger, inner membrane protein with GTPase activity (Kammler et al., 1993; Marlovits et al., 2002). Downstream of *feoB* is a small ORF, termed *feoC* (*yhgG*), whose function is not known (Hantke, 2004). *V. cholerae* FeoA and FeoB each have 40% amino acid identity with FeoA and FeoB of *E. coli*, respectively (Wyckoff et al., 2006). *V. cholerae feoC* initiates with a GTG codon that overlaps the stop colon of *feoB* (Fig. 1). The predicted *V. cholerae* FeoC protein is the same size as *E. coli* FeoC, but the two proteins have only 11% amino acid identify. A clone containing the *V. cholerae feoABC* genes was tested for its ability to promote iron acquisition by *Shigella flexneri* strain SM193w. SM193w (*sitA*, *feoB*, *iucD*) has no functional system for the transport of free iron and fails to grow on LB agar in the absence of a usable siderophore such as aerobactin (Fig. 3) (Runyen-Janecky et al., 2003). Both the *E. coli* and *V. cholerae feo* genes stimulated growth of SM193w on agar lacking supplemental aerobactin, suggesting that both systems promote iron transport (Fig. 3A). The *V. cholerae feoABC* genes also promoted the transport of radioactive iron into SM193w. The transport of iron by Feo was stimulated by the addition of the reducing agent ascorbate (Wyckoff et al., 2006), consistent with previous work indicating that Feo transports ferrous iron.

ABC Transporters

The *V. cholerae* genome contains several genes encoding ABC transport systems that are annotated as ferric iron transporters. One of these systems, termed FbpABC, consists of a periplasmic binding protein, a permease protein and an ATPase (Fig. 1). The FbpABC proteins have about 50% amino acid identity with FbpABC of

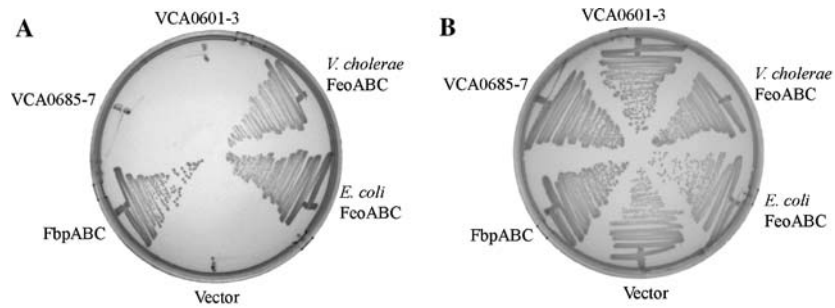

Fig. 3 Growth of the *S. flexneri* strain SM193w. SM193w carrying genes for the indicated iron transport systems were streaked on either (A) LB agar or (B) LB agar supplemented with an aerobactin-containing cellular supernatant. The genes for the indicated iron transport systems were supplied on plasmids from Wyckoff et al. (2006), reprinted with permission

Mannheimia haemolytica, the most closely related system for which functional data are available (Kirby et al., 1998). *M. haemolytica* FbpA has been crystallized (Shouldice et al., 2003, 2004), and the amino acids that coordinate the ferric iron, as well as those that bind a synergistic anion, are all conserved in *V. cholerae* FbpA. A clone containing *V. cholerae fbpABC* stimulated growth of *S. flexneri* strain SM193w on LB agar (Fig. 3A) and promoted the transport of radioactive iron into SM193w. Unlike Feo, transport of iron by Fbp was inhibited by ascorbate, indicating that FbpABC is likely to be a ferric iron transporter (Wyckoff et al., 2006).

Two other sets of genes for potential ABC transporters, (VCA0685–0687) and (VCA0601–0603), are annotated as ferric iron transporters. These genes, however, were not regulated by iron or Fur (Mey et al., 2005b), and plasmids encoding these systems failed to stimulate the growth of *S. flexneri* SM193w (Fig. 3A) (Wyckoff et al., 2006). Thus, there is no evidence that these systems function in iron transport.

Additional transporters

To determine whether all of the ferric and ferrous iron transport systems of *V. cholerae* have been identified, strains carrying a mutation in the vibriobactin synthesis gene *vibB*, in *feoB*, in *fbpA* or in combinations of these genes were constructed. The size of the colonies formed by these strains was then determined on LB agar, a condition in which neither heme nor xenosiderophores are expected to be available. The colony size of the *vibB* mutant was the same as the wildtype, except when the iron chelator EDDA was added to the medium (Fig. 4). The *vibB*, *feoB*, *fbpA* triple mutant formed colonies that were smaller than the wildtype and *vibB* single mutant at all iron levels tested. However, this strain still formed colonies in the presence of EDDA, indicating that at least one functional high affinity iron transport system, not involving heme or xenosiderophores, is present in this strain (Wyckoff et al., 2006). No candidates for additional iron transporters have been identified in the *V. cholerae* genome sequence. No genes with significant similarity to *sitABCD*

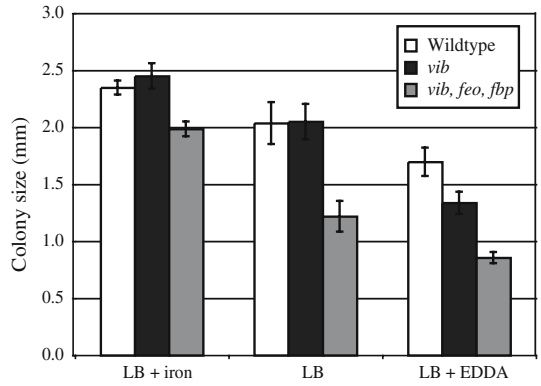

Fig. 4 Growth of *V. cholerae* iron transport mutants. Bacteria were spread on LB agar or on LB agar containing either 40 μM $FeSO_4$ or 5 μg/ml EDDA. The diameter of the colonies was measured after incubation at 37°C for 24 h. The error bars indicate one standard deviation

(Zhou et al., 1999; Kehres et al., 2002), *mntH* (Makui et al., 2000), *corA* (Hantke, 1997), *zupT* (Grass et al., 2005) or *efeU* (*ycdN*) (Große et al., 2006) are present in the genome, suggesting that there may be an additional class of iron transporters.

Location of iron transport genes in the *V. cholerae* genome

The *V. cholerae* genes are nonrandomly distributed between the two chromosomes. Most genes for housekeeping functions, as well as the major virulence factors, are located on the larger chromosome (chromosome 1). The smaller chromosome (chromosome 2) has only a limited number of essential genes, and contains a high proportion of ORFs that appear to have been acquired by horizontal gene transfer (Heidelberg et al., 2000; Schoolnik and Yildiz, 2000). Consistent with this observation, the iron acquisition genes that are most highly conserved in enteric bacteria are located on chromosome 1. These genes, which include those for siderophore biosynthesis, the TonB2 system, IrgA, ferrichrome transport, and the Feo system, are likely derived from ancestral genes for the enteric bacteria (Fig. 1).

In contrast, all of the genes for heme transport and utilization are located on the small chromosome (Fig. 1). Except for *hasR*, most of the heme

transport genes are conserved within the Vibrionaceae, suggesting that they were acquired before the divergence of these lineages. The *vctAPDGC* enterobactin transport genes, which are not closely related to those from enterics, may be recently acquired in the *V. cholerae* lineage (Mey et al., 2002).

Regulation

Because iron is an essential nutrient, but toxic at high concentrations, the genes for iron acquisition are regulated in response to iron availability. In *V. cholerae*, as in most gram negative bacteria, this regulation is mediated primarily through the action of the Fur protein (Litwin et al., 1992; Mey et al., 2005b). In iron replete conditions, ferri-Fur binds to the promoter of iron-regulated genes, preventing their expression. In low iron, Fur is present in the iron-free form, which does not bind to the regulated promoters (Bagg and Neilands, 1987). A consensus sequence has been derived for the Fur binding site in *E. coli*, and this is referred to as an iron or Fur box.

Microarrays analysis was performed with wild-type classical strain O395 in high and low iron conditions. The wildtype was also compared with a *fur* mutant during growth in iron replete conditions (Mey et al., 2005b). The growth conditions were carefully controlled, so that the growth phase and growth rates of the strains being compared were similar. Under these conditions, nearly all of the genes involved in iron acquisition were negatively regulated by iron and Fur, as expected. As observed in *E. coli*, the *sodA* and *fumC* genes were both repressed by iron and Fur, but unlike *E. coli*, both the iron storage genes *bfd* and *bfr* were also negatively regulated (McHugh et al., 2003; Mey et al., 2005b). Approximately 20 ORFs of unknown function were repressed by iron and Fur, and about half of these appear to be in operons with known iron-regulated genes. These genes may encode as yet unknown functions in iron acquisition or virulence.

The promoters of most of the *V. cholerae* Fur-regulated operons contained sequences with similarity to the *E. coli* Fur box consensus. In two cases, the binding of Fur to this site was confirmed by footprinting (Watnick et al., 1998; Butterton et al., 2000), and gene fusions have localized the promoter regions responsible for regulation by Fur in several other promoters (Mey et al., 2005a). A consensus sequence has been determined for *V. cholerae* Fur regulated genes, and as expected, it is similar to the *E. coli* Fur box consensus (Mey et al., 2005b).

It was recently discovered that *E. coli* Fur represses the negative small regulatory RNA RyhB, and the net effect is an apparent positive regulation of certain genes by Fur (Masse and Gottesman, 2002). RyhB has also been identified in *V. cholerae*, and its regulon was characterized through microarray analysis of a *ryhB* mutant and also a strain over-expressing *ryhB* (Davis et al., 2005; Mey et al., 2005a). Similar to *E. coli*, *V. cholerae* RyhB repressed the synthesis of SodB and a variety of iron-containing proteins, many of which are involved in TCA and electron transport. *V. cholerae* RyhB also regulated genes not known to be regulated by RyhB in *E. coli*, including those for motility and chemotaxis, indicating there are both shared and species-specific members of the RyhB regulon. At 214 nucleotides, *V. cholerae* RyhB is more than twice the length of *E. coli* RyhB (90 nucleotides), and these additional sequences may contribute to its expanded regulon (Davis et al., 2005; Mey et al., 2005a)

Vibrio cholerae fur mutants grow poorly and rapidly picked up mutations that suppressed the growth defect. We recently determined that two independently-derived spontaneous suppressor mutants carried mutations that eliminated expression of *ryhB*, indicating that unregulated expression of *ryhB* is detrimental (AR Mey, unpublished observations). To control for this, only phenotypes that could be complemented with the *fur* gene, either supplied on a plasmid or as a chromosomal ''knock-in'', were considered to be due to the *fur* mutation.

Unexpectedly, expression of *tcp* genes was reduced in the *fur, ryhB* mutant, but *tcp* expression was not significantly influenced by iron levels. This mutant also failed to agglutinate, a process that requires synthesis and assembly of the Tcp pilus. This phenotype was complemented by *fur* and was not observed in a either a *ryhB* mutant or in a strain overproducing *ryhB* (Mey

et al., 2005b). Reduced Tcp expression may contribute to the reduced colonization of the infant mouse intestine by the *fur* mutant as described below.

In the N16961 genetic background, a *V. cholerae ryhB* mutant had a reduced ability to form biofilms, and this defect was largely suppressed by supplementing the medium with excess iron or succinate. The *ryhB* mutant also exhibited a lower frequency of switching from the smooth to the rugose phenotype on low nutrient agar and reduced chemotactic motility. How RyhB exerts its effect on these processes is not understood (Mey et al., 2005a).

In addition to the Fur and RyhB global regulators, the expression of several iron acquisition genes was also influenced by gene-specific regulators. The best characterized of these is *irgA*, which requires the divergently transcribed LysR family regulatory gene *irgB* for its expression. Both *irgA* and *irgB* are repressed by Fur. In low iron, Fur is released from the *irgA-irgB* intergenic region, relieving repression of both genes. However, *irgA* is only expressed when IrgB is bound to the promoter region at a site distinct from the Fur box (Goldberg et al., 1991; Watnick et al., 1998). IrgB does not appear to regulate any genes in addition to *irgA* and itself (Watnick et al., 1998) and V. Kanukurthy, unpublished data). The effect of this dual regulation may to be to amplify the response. Microarray analysis showed that *irgA* was among the most highly induced genes in response to low iron. Other highly regulated genes included *hutA* and *vctA*, which are divergently transcribed from predicted LysR- and AraC-type regulators, respectively. The only other genes with similar high levels of induction were in operons preceded by multiple Fur boxes (Mey et al., 2005b).

Virulence

It has been difficult to determine the iron sources that are used by *V. cholerae* during infection of its host. The most frequently used animal model is the infant mouse. In competition assays, neonatal mice are inoculated intragastrically with equal numbers of a mutant and parental strain, and the ratio of viable mutant cells to parental cells in the intestine after 24 h is determined. The competitive index (the output ratio normalized to the input ratio) is then calculated (Taylor et al., 1987). If the mutant and parent colonize the intestine equally well, the competitive index is one.

A summary of competition experiments with various *V. cholerae* mutants is presented in Fig. 5. These experiments were done at different times and are not all in the same genetic background, but they do summarize our current understanding of the role of *V. cholerae* iron acquisition genes in this assay. Mutations in *tonB1* or *tonB2* only moderately reduced colonization, and the colonization defect in the *tonB1*, *tonB2* mutant was more severe. This suggested that one or more TonB-dependent receptors is required for optimal colonization (Seliger et al., 2001).

Mutants defective in a single gene or iron transport system were also tested. An *irgA* mutant was previously reported to be highly attenuated, leading to the model that *irgA* is an important virulence factor (Goldberg et al., 1992). A newly constructed mutant in the same genetic background was not at a significant competitive disadvantage compared with the wildtype (Fig. 5) and was not attenuated in an LD_{50} assay (Mey et al., 2002), indicating that the virulence defect of

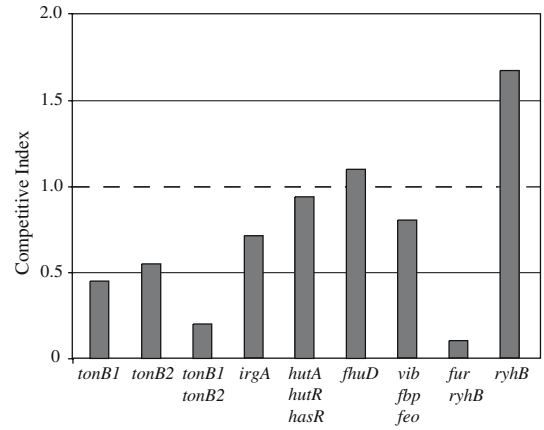

Fig. 5 Competitive index of *V. cholerae* mutant strains. The competitive index for *V. cholerae* strains carrying the indicated mutations was determined in infant mice by the method of Taylor et al. (1987). The references for these experiments are given in the text

the original strain may be caused by a secondary mutation. Strains defective in heme utilization (Mey and Payne, 2001) or in ferrichrome transport (C. Fisher, unpublished data) also competed well with their parent strain. Strains carrying defects in vibriobactin transport and biosynthesis have not been tested in competition assays but do not have highly reduced ability to colonize or proliferate in the intestines of infant mice (Sigel et al., 1985; Henderson and Payne, 1994a) or rabbits (Tashima et al., 1996). Since none of the strains with mutations in a single receptor gene appear to be attenuated, it is unclear whether the defect observed with the *tonB1*, *tonB2* double mutant is due to multiple iron transporters functioning in the wildtype or whether TonB activity is required for a function other than its role in iron transport. A mutant defective in all of the known ferric and ferrous iron acquisition systems (*vibB*, *fbpA* and *feoB*), but retained the ability to transport heme and the xenosiderophores, competed well with its parent. Since it is not anticipated that heme or xenosiderophores would be available in the infant mouse intestine, this is additional evidence that an unidentified iron transport system is present in *V. cholerae* (Wyckoff et al., 2006).

The *fur* mutant grows too slowly to test in the competition assay, but the *fur*, *ryhB* double mutant, which grows in vitro at a rate comparable to the wild-type strain, competed poorly in the mouse model (Fig. 5). This defect was not due to the *ryhB* mutation, since it was complemented by the *fur* gene in a chromosomal ''knock-in'', and because the *ryhB* single mutant competed well with its wildtype parent (Fig. 5) (Mey et al., 2005a, 2005b). Poor colonization by the *fur* mutant may well be due to its defect in proper expression of the *tcp* locus genes.

Summary and future directions

Vibrio cholerae can transport siderophores and heme, as well as ferric and ferrous iron. Much remains to be learned about the currently identified systems. How does Feo work? How is vibriobactin exported after its synthesis? Is stimulation of Tcp synthesis by Fur a direct effect, and if not, how is this working? Why does the *ryhB* mutant have a biofilm defect? What is the role of HutZ in heme utilization? Is a heme degrading enzyme present in *V. cholerae*?.

Analysis of strains carrying mutations in the known iron transport system genes indicate that at least one additional system is present. Since no other potential iron transporters have been identified from analysis of the genome sequence, genetic approaches may be needed to identify the genes for these systems. The presence of multiple iron transport systems may reflect the overall importance of iron, and the need to optimize its acquisition in the various environments encountered by *V. cholerae*. The contribution of the different iron acquisition systems in these environments is an important area for future study.

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