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The heme-uptake gene cluster in *Vibrio fischeri* is regulated by Fur and contributes to symbiotic colonization

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Summary

Although it is accepted that bacteria colonizing host tissues are commonly faced with iron-limiting conditions and that pathogenic bacteria often utilize iron from host-derived heme-based compounds, the mechanisms of iron acquisition by beneficial symbiotic bacteria are less clear. The bacterium *Vibrio fischeri* mutualistically colonizes the light organ of the squid *Euprymna scolopes*. Genome sequence analysis of *V. fischeri* revealed a putative heme-uptake gene cluster, and through mutant analysis we confirmed this cluster is important for hemin use by *V. fischeri* in culture. LacZ reporter assays demonstrated Fur-dependent transcriptional regulation of cluster promoter activity in culture. GFP-based reporter assays revealed that gene cluster promoter activity is induced in symbiotic *V. fischeri* as early as 14 h post-inoculation, although colonization assays with the heme uptake mutant suggested an inability to uptake heme does not begin to limit colonization until later stages of the symbiosis. Our data indicate that the squid light organ is a low iron environment and that heme-based sources of iron are used by symbiotic *V. fischeri* cells. These findings provide important additional information on the availability of iron during symbiotic colonization of *E. scolopes* by *V. fischeri*, as well as the role of heme uptake in non-pathogenic host-microbe interactions.

Introduction

Pathogenic bacteria associated with animal or plant host tissues often face an iron-limited environment, and must use specific acquisition systems to obtain this essential element. Such iron scavenging is especially well studied in pathogenic bacteria that infect vertebrates. Although these bacteria can possess uptake systems for ferrous iron, transferrin, lactoferrin, and/or ferritin (Wandersman & Delepelaire, 2004), one of the most abundant iron sources in vertebrate tissue is heme-based (Anzaldi & Skaar, 2010). As a result, many pathogenic bacteria have specialized systems to facilitate heme uptake and utilization (Anzaldi & Skaar, 2010). For example, heme uptake systems have been described in fish pathogens among the

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Vibrionaceae, and in certain cases these systems may be expressed and play a role in iron uptake during infection (Lemos & Osorio, 2007). Regulation of the heme uptake system genes has been described in *Vibrio anguillarum*, where expression is controlled in response to iron concentration through the activity of the ferric uptake regulation (Fur) protein (Mourino *et al.*, 2006).

In contrast to pathogenic host-microbe interactions, relatively little is known about the role of heme uptake in beneficial relationships of bacteria with their hosts. Animals and plants often support the growth and persistence of mutualistic bacteria in specific specialized tissues, and for the most part it remains unclear whether the bacteria supported by the host in these environments face the same iron limitations and sources as pathogens invading these or other tissues. With the exception of the interactions between *Bradyrhizobium japonicum* and soybean plants (Nienaber *et al.*, 2001), and *Sodalis glossinidius* and the tsetse fly (Runyen-Janecky *et al.*, 2010), the role of microbial heme uptake in mutualistic symbioses has not been explored. In the case of *B. japonicum*, disrupting bacterial heme uptake does not affect symbiotic nitrogen fixation, suggesting that heme is not a required iron source for development or maintenance of the symbiotic relationship between the bacterium and plant (Nienaber *et al.*, 2001). In contrast, *in vivo* expression analysis demonstrated that portions of the heme uptake system of *S. glossinidius* are expressed during insect colonization, suggesting that a heme-based iron source could be important for the interactions between the bacterium and invertebrate host (Runyen-Janecky *et al.*, 2010).

Vibrio fischeri is a bioluminescent marine bacterium that forms a beneficial symbiotic relationship with the Hawaiian bobtail squid *Euprymna scolopes*, during which the bacteria colonize a specific organ in the animal called the light organ (Visick & Ruby, 2006). *V. fischeri* grows rapidly during the first day of light-organ infection, doubling as rapidly as every 30 minutes (Ruby & Asato, 1993); however, it has also been reported that the symbionts may experience iron-limited growth in the light organ later in infection (Graf & Ruby, 2000). The squid vent a subpopulation of symbionts each day, with the residual bacteria regrowing (Boettcher, 1996), and it is possible that *V. fischeri* must actively scavenge some form of iron to persistently colonize the host through these cycles of regrowth. Based on genome sequence analysis, *V. fischeri* appears to possess multiple types of iron acquisition systems similar to those in pathogenic bacteria, including a predicted heme-uptake system (Ruby *et al.*, 2005). Although a detailed analysis of iron source availability in the host has not been performed, it has been suggested that free ferrous or ferric iron is not the main form of iron present for *V. fischeri* in the light organ environment (Wang *et al.*, 2010), and that iron may be acquired by the bacterium from a heme-based source during host association (Wang *et al.*, 2010, Wier *et al.*, 2010).

To investigate the role of heme uptake in *V. fischeri*, we used mutant analysis to test and confirm that the genome-predicted heme-uptake gene cluster is important for hemin uptake and/or utilization in *V. fischeri*, and to explore the role of heme uptake during symbiotic colonization. These studies were combined with reporter-based analyses to characterize transcriptional regulation of the genes of the heme uptake cluster during growth both in culture media and in the host light organ. The results of this study not only advance our understanding of the role of iron acquisition in *V. fischeri* during host colonization, but

provide further insight into how heme acquisition may contribute to beneficial host-microbe interactions in general.

Results

Identification of a heme-uptake gene cluster in *V. fischeri* ES114

Analysis of the *V. fischeri* ES114 genome sequence (Ruby *et al.*, 2005) indicated the presence of a gene cluster (ORFs VF_1220-VF_1228) with similar gene content and arrangement as compared to heme-uptake cluster genes in other members of the *Vibrionaceae* (Lemos & Osorio, 2007) (Fig. 1, Table 2). Based on this similarity, we predicted that these *V. fischeri* genes encode proteins involved in providing energy for the transport process (TonB, ExbB, ExbD), a periplasmic heme-binding protein (HutB), an inner membrane permease (HutC), and an ABC-transporter ATPase (HutD) (Table 2). This gene cluster also encodes the proteins HutW, HutX, and HutZ. HutW is a member of the radical SAM superfamily (Sofia *et al.*, 2001), and is often annotated as a coproporhyrinogen oxidase (as in *V. fischeri* ES114) due to its homology to HemN. However, the *hutW* from *Vibrio cholerae* was unable to complement a *Salmonella enteric* serovar Typhimuruim *hemN* mutant (Wyckoff *et al.*, 2004), suggesting that HutW is not a coproporphyrinogen oxidase. As a result, the specific function of HutW remains undefined. The *V. fischeri hutX* and *hutZ* genes are annotated as proteins of unknown function; however, it has been shown that HutZ in *V. cholerae* and the homologous HuvZ in *V. anguillarum* are required for optimal heme utilization (Mourino *et al.*, 2004, Wyckoff *et al.*, 2004). Similar to many of the *Vibrionaceae*, a gene encoding a putative TonB-dependent heme receptor protein (VF_1234, VF_A0332) is not directly linked to the *V. fischeri* VF_1220-VF_1228 cluster (Lemos & Osorio, 2007), and therefore the contribution of heme receptor proteins was not explored in this study. To determine whether proteins encoded by the VF_1220-VF_1228 cluster were necessary for use of hemin as a sole iron source by *V. fischeri*, we constructed mutant strain AKD910, which contains an in-frame deletion of VF_1220-VF_1228.

AKD910 is unable to use hemin as a sole iron source

Growth of AKD910 (lacking VF_1220-VF_1228) was compared to that of wild type in iron-limited mineral-salts medium either in the absence or presence of hemin. Growth density assays demonstrated that AKD910 was unable to use hemin as a sole iron source (Fig. 2), indicating that this gene cluster encodes proteins necessary for heme uptake and/or utilization as an iron source. However, when ferrous sulfate was provided as an iron source, both AKD910 and wild-type cultures grew to similar final optical densities of 1.29 +/− 0.01 and 1.28 +/− 0.02, respectively.

Promoters for the gene cluster are repressed by Fur and induced in response to low iron

Plasmid-based promoter-*lacZ* fusions were constructed to determine the influence of iron levels on heme-uptake gene promoter activity. The organization of this gene cluster in *V. fischeri* (Fig. 1) suggested that the genes are expressed in two transcriptional units. Two promoter-*lacZ* fusion plasmids were constructed by directionally cloning DNA fragments corresponding to the sequence upstream of VF_1225 and VF_1226. These plasmids were then moved via conjugation into the appropriate *V. fischeri* strains.

Comparative analysis of expression patterns between the two reporters in various strains and culture conditions indicated congruent promoter activities (Fig. 3A and B). Promoter activity in wild-type cells was relatively low in LBS, an iron-replete rich medium; however, promoter activity was approximately 30-fold higher in LBS medium when iron chelator was added (Fig. 3A). This influence of iron levels on promoter activity, and a putative Fur box in the VF_1225 upstream sequence (Wang *et al.*, 2010), suggested that the *V. fischeri* homolog of the ferric uptake regulator repressor protein (Fur, VF_0810) could influence VF_1225 promoter activity. To test this possibility, we introduced the reporter plasmids into a *V. fischeri* strain lacking *fur* (YLW111), and as described above, assayed for promoter activity both in the presence and absence of iron chelator (Fig. 3B). These data demonstrate that *V. fischeri* Fur mediates repression of promoter activity in response to iron availability.

Although promoter activity increases under low-iron conditions, there was the possibility that activity could also be influenced by the addition of hemin to the assays. Our results suggest that under the conditions tested, promoter activity does not increase in the presence of hemin (Fig. 3A). However, a trend of a decrease in promoter activity in wild-type cells grown in medium amended with hemin and an iron chelator as compared to iron chelator alone was observed (Fig. 3A), although it was not always statistically significant. We hypothesized that this trend was due to the processing of hemin by the proteins encoded in the gene cluster, effectively increasing intracellular iron levels, resulting in moderate Fur-mediated repression of promoter activity. When promoter activity levels were measured under identical growth conditions (LBS medium amended with iron chelator, with or without hemin) in the mutant strain AKD910, which lacks the ability to utilize hemin as an iron source, no significant decrease in promoter activity was observed in the presence of hemin (data not shown). These data support the hypothesis that products of this gene cluster are involved in processing of hemin as an iron source, and the corresponding increase in intracellular iron is sensed by Fur in wild-type cells.

Heme uptake gene expression is induced during symbiotic colonization

To determine whether gene cluster expression is induced during colonization of the host, P_{VF_1225}-*gfp* and P_{VF_1226}-*gfp* reporter plasmids, which also encode a constitutively expressed red fluorescent protein gene (*rfp*), were constructed and introduced into wild-type *V. fischeri*. Juvenile aposymbiotic squid colonized by these strains were monitored using epifluorescence microscopy. Similar results were obtained with the two reporters, therefore only P_{VF_1225}-*gfp* (pAKD911) data are shown. Results of these assays demonstrated that *V. fischeri* colonizing the juvenile squid light organ are expressing the gene cluster at 28 h post-inoculation (Fig. 4C and D), with similar results obtained at earlier (14 h) and later (48 h, 72 h) timepoints (data not shown). Similar to the β-galactosidase assays, measureable GFP expression is only observed in culture when iron is limiting (Fig. 4E–H), suggesting that the genes are expressed in response to low iron conditions present in the squid light organ.

The heme-uptake genes influence *V. fischeri* symbiotic competency

The expression of the gene cluster during host colonization indicated that *V. fischeri* could be using host-derived heme compounds as a source of iron. To determine whether the ability to utilize heme was important for host colonization, we assayed the ability of AKD910

(ΔVF_1220-1228) to colonize aposymbiotic juvenile squid. In experiments where *E. scolopes* squid were exposed to clonal inocula containing either AKD910 or the wild type, colonization levels were similar in the two treatments at time points 48 and 72 h post-inoculation, suggesting that AKD910 does not have a detectable symbiotic defect compared to the wild type (data not shown). However, it has been documented that symbiotic defects in certain mutants are only apparent when they are competed with the wild type in mixed inocula (Stabb & Ruby, 2003, Visick & Ruby, 1998). Competitive colonization assays revealed AKD910 was less competitive for colonization (average relative competitive index or RCI value of less than one, see materials and methods), but this deficiency was not observed until several days post-inoculation, and became increasingly robust at later time points (Fig. 5). These results indicate that although promoter activity is induced in symbiotic *V. fischeri* as early as 14 h post-inoculation, an inability to transport and use heme does not detectably limit colonization competitiveness until 48 h post-inoculation, with the most significant effects at later stages of the symbiosis (~96 h).

Discussion

Bacterial pathogens often encounter iron-limiting conditions in hosts, and must employ iron-scavenging strategies for successful infection (Weinberg, 1978). Although there are numerous forms of iron that these bacteria could acquire during host colonization, heme in particular has been shown to influence bacterial virulence, and strains defective in heme uptake demonstrate reduced virulence (reviewed in (Lee, 1995)).

In contrast, the role of heme as an iron source in beneficial host-microbe interactions is less clear. Bacterial growth in mutualistic symbioses is often supported by the host, making it uncertain whether these bacteria also face similar iron-limited host environments, and, if so, the role of iron limitation in the relationship. Previous studies of the *V. fischeri-E. scolopes* mutualistic symbiosis suggested that the squid light organ may be an iron-limited environment after the first day of infection (Graf & Ruby, 2000), and that heme-based compounds could be important sources of iron for symbiotic *V. fischeri* (Wang *et al.*, 2010, Wier *et al.*, 2010). Based on analysis of the *V. fischeri* genome sequence (Ruby *et al.*, 2005) and identification of a putative heme-uptake gene cluster (Fig. 1) similar to those characterized in other *Vibrionaceae* (Lemos & Osorio, 2007), we hypothesized that this gene cluster would be expressed in response to iron limitation and that an ability to uptake and utilize heme-based iron would be important for *V. fischeri* during the symbiotic lifestyle.

Our experimental results confirm that the identified gene cluster is involved in hemin uptake and utilization, and that regulation of gene expression under culture conditions is similar to other members of the *Vibrionaceae* that form pathogenic relationships with multicellular hosts (Lemos & Osorio, 2007, Mourino *et al.*, 2006). The idea that pathogenic and mutualistic members of the *Vibrionaceae* have nearly identical uptake systems that are regulated similarly suggest that, although the outcome of the host-bacterial relationships are very different, there are fundamental similarities in how these bacteria obtain host-derived iron sources. Further understanding of these similarities and differences will provide useful insight into what defines pathogenesis vs. mutualism.

Beyond providing a comparison of culture-based heme-uptake gene regulation in pathogenic and mutualistic *Vibrionaceae*, our results provide important insight into how iron influences the symbiotic relationship between *V. fischeri* and *E. scolopes*. Fluorescence-based reporter assays during symbiotic colonization provide strong support for the idea that the squid light organ is an iron-limited environment (Fig. 4). Results from culture-based reporter assays indicate that gene cluster expression is induced in response to low iron conditions through the regulatory activity of Fur, and squid colonization assays demonstrate that the same reporter constructs are induced during colonization (Figs. 3 and 4). In combination, these experiments provide data to support the model of the light organ being an iron-limited environment, as previously suggested (Graf & Ruby, 2000, Wang *et al.*, 2010), although there may be other currently undescribed conditions and regulators that influence gene cluster expression during host colonization.

In addition, our results indicate that heme uptake and/or utilization does play a role in the establishment of a successful symbiotic relationship (Fig. 5), providing further information concerning the role of this physiological process in beneficial host-microbe interactions. One interesting observation from these analyses is that although the promoters are active at early time points post-inoculation, colonization is not measurably limited in a strain lacking the gene cluster (AKD910) until later stages of the symbiosis (48–100 h) (Figs. 4 and 5). This apparent discrepancy could, in part, be explained by what is currently known about host physiology during symbiotic colonization.

The levels of heme-based iron available to host-associated *V. fischeri* could be related to morphological changes that occur in the host epithelium lining the light organ crypts during the day-night cycle of the symbiosis. The animals use the light produced by bacteria during nocturnal behaviors, and at dawn expel approximately 90% of the bacterial population, the remainder of which will divide and recolonize the light organ (Nyholm & McFall-Ngai, 2004). Corresponding with these daily ventings, the epithelial cells bleb into the interior of the light organ (Wier *et al.*, 2010). These blebs could be a source of heme-based iron, and this is reflected in the transcriptional response of the bacterial population to this stage of the symbiosis, which indicates an increase in transcript levels for members of the heme-uptake genes (Wier *et al.*, 2010). These changes were documented in adult animals with a “mature” symbiosis, and it is likely that during the onset of colonization (prior to 94 h) the morphological changes to the host epithelium are less pronounced during the day-night cycle, resulting in less heme-based iron available to the bacteria. In support of this hypothesis, squid colonization data using heme biosynthesis mutants suggest heme levels are too low to support the growth of such auxotrophs in the light organ during the initiation of colonization (unpublished data). Currently there are efforts underway to develop techniques to reliably raise juvenile squid past 96 h (M.J. McFall-Ngai and E.G. Ruby, personal communication), and in the future it may be possible to study the contribution of heme acquisition to the symbiosis from the juvenile stage to adulthood.

The results of this study also raise the question as to why the host would temporally limit iron availability to *V. fischeri*. One possibility is that iron availability helps to modulate growth rate of the bacteria. For example, if heme-based iron is made available in limited quantities to the residual bacteria present after venting, this iron could be used to promote

controlled growth and recolonization of the light organ in preparation for the animal's nocturnal activity. In addition, iron limitation may influence the onset of light production by the bacteria. Previous reports identified that production of luminescence is repressed under certain iron-replete conditions, and the presence of an iron chelating compound in liquid culture induces luminescence of some strains of *V. fischeri* at lower cell densities (Dunlap, 1992, Haygood & Nealson, 1985). Experiments designed to identify the molecular mechanism of iron influence on bioluminescence were performed in transgenic *E. coli* carrying the *lux* genes (Dunlap, 1992), yet it remains unclear how iron is physiologically linked to these phenotypes in *V. fischeri*. However, since this earlier publication, genetic tools have been constructed for *V. fischeri* that will facilitate future studies to characterize the link between iron levels, luminescence, and successful colonization and maintenance of the symbiosis.

This study represents the first genetics-based investigation of heme-based iron acquisition in *V. fischeri*, and this information in combination with previous and future studies can be used to further define the parameters important for successful establishment and maintenance of the mutualistic symbiosis between *V. fischeri* and *E. scolopes*. As this system is an important model for beneficial host-microbe interactions, further understanding of this relationship provides an important comparison to pathogenic host-bacterial interactions and contributes to the ultimate goal of defining the boundaries between pathogenesis and mutualism.

Experimental Procedures

Growth media

V. fischeri strains were grown at 28 °C (unless noted) in either LBS (Stabb *et al.*, 2001), SWT (Boettcher & Ruby, 1990) wherein seawater was replaced with Instant Ocean (Aquarium Systems; medium referred to as ASWT), or mineral salts medium (per liter: 378 μl 1M $NaPO_4$ (pH 7.5), 50 ml 1M Tris (pH 8.0), 0.59 g NH_4Cl, 3 mg $FeSO_4$-$7H_2O$, 13.6 g $MgSO_4$-$7H_2O$, 0.83 g KCl, 19.5 g NaCl, 1.62 g $CaCl_2$-$2H_2O$) containing 40 mM glycerol and one g l-1 vitamin-free casamino acids (Difco) as carbon and nitrogen sources. When indicated, 100 μg ml^{-1} kanamycin or 5 μg ml^{-1} erythromycin were added to the culture medium. *Escherichia coli* strains were grown at 37 °C in LB medium (Miller, 1992) with 40 μg ml^{-1} kanamycin or 22.5 g l^{-1} HiVeg special infusion (HIMEDIA) containing 150 μg ml^{-1} erythromycin.

Strains and plasmids

Strains and plasmids used in this study are listed in Table 1. General cloning procedures were used in *E. coli* strains DH5α (Hanahan, 1983) or DH5α*λpir* (Dunn *et al.*, 2005), and constructs containing the RP4 origin of transfer were introduced into *V. fischeri* using triparental mating (Stabb & Ruby, 2002). All *Vibrio* plasmids used in this study contain the pES213 origin of replication, which is maintained at approximately 10 copies per chromosome (Dunn *et al.*, 2005). Primer sequences are available upon request.

Construction of *V. fischeri* mutant strains

A strain lacking the putative hemin uptake and processing genes (VF_1220-VF_1228), which are divergently arranged at one locus (Fig. 1), was constructed using allelic exchange (Bose *et al.*, 2008), resulting in an in-frame deletion mutant (AKD910). Briefly, approximately 1.6 kb of DNA downstream of VF_1220 was PCR-amplified and fused to an approximately 1.6-kb DNA fragment downstream of VF_1228 using an engineered *Nhe*I 6-bp restriction site. Additional codons were included in order to design specific primers with reasonable G+C content, resulting in inclusion of the last six codons of VF_1220 and the last four codons of VF_1228. Due to the large size of the gene cluster (~10 kb) plasmid-based complementation methods were not used with AKD910; however, given the gene arrangement (Fig. 1) and the complete deletion of the cluster, polar effects of the deletion are not expected.

A strain lacking *fur* (VF_0810; strain name YLW111) was constructed using an identical approach, with inclusion of the first seven codons of VF_0810.

Plasmid-based P_{VF_1225} and P_{VF_1226} transcriptional reporters

The plasmid-based reporters pAKD911 (P_{VF_1225}-*gfp*) and pAKD912 (P_{VF_1225}-*lacZ*) were constructed by PCR amplifying a 243-bp fragment that included 221 bp upstream of the start codon of VF_1225 (*tonB*; Fig. 1), and directionally cloning this fragment into pVSV209 or pAKD701 respectively, whereas pAKD913 (P_{VF_1226}-*gfp*) and pAKD914 (P_{VF_1226}-*lacZ*) were constructed by PCR amplifying a 576-bp fragment that included 499 bp upstream of the start codon of VF_1226 (*hutW*; Fig. 1) and directionally cloning this fragment into pVSV209 or pAKD701 respectively.

Growth of wild-type *V. fischeri* and AKD910 using hemin as an iron source

To assay growth of *V. fischeri* using hemin as an iron source, cultures of wild type and AKD910 were grown overnight in LBS medium and diluted 1000-fold into mineral salts medium lacking $FeSO_4$ and supplemented with 50 μM of the iron chelator 2,2′-bipyridyl (Sigma, St. Louis, MO), either with or without 77 μM (50 μg ml^{-1}) bovine hemin (Sigma) or 10.8 μM ferrous sulfate. The diluted cultures were grown in 200 μl aliquots in a Falcon polystyrene 96-well plate (Becton Dickinson, Franklin Lakes, NJ) for 30 h at 28°C. Growth was measured by absorbance at 595 nm using a BioTek Synergy 2 plate reader, and absorbance readings (optical density) were adjusted to a 1 cm path length. Each experiment included two independent cultures of each strain grown in duplicate, and the experiment was repeated three times. One representative experiment is shown.

β-galactosidase assays of promoter activity

Overnight cultures of wild type or YLW111 (Δ*fur*) containing either the control plasmid pAKD701 or the heme uptake reporter plasmids pAKD912 or pAKD914 were grown in LBS medium containing kanamycin. Cultures were diluted 1000-fold into LBS medium containing kanamycin, either with or without 100 μM of the iron chelator 2,2′-bipyridyl and/or 31 μM hemin, and grown to an OD_{600} of between 0.4 and 0.5. Cells were harvested by pelleting two ml of culture at 15,000 × g for 5 minutes. Supernatant was discarded and

pellets frozen at −20 °C for no longer than 24 hours. Cell pellets were resuspended in one ml of Z buffer and β-galactosidase assays performed using a modified Miller assay as previously described (Bose *et al.*, 2008). The average Miller unit values for the strains containing pAKD701 were subtracted from the corresponding pAKD912 or pAKD914 individual sample Miller unit values prior to calculating averages to account for effects of medium amendments on absorbance readings. For each experiment three independent cultures of each strain were assayed, and each experiment was repeated at least three times. Data shown is from one representative experiment.

Squid colonization and reporter assays

For all squid colonization assays, *V. fischeri* strains were grown in ASWT medium at 28°C without shaking to an OD_{595} of between 0.3 and 0.6 and diluted in Instant Ocean to between 1360 and 2600 CFU ml^{-1} inocula. Aposymbiotic juvenile squid were exposed to inocula for ~15 h before being transferred to fresh filter-sterilized Instant Ocean.

For RFP- and GFP-based reporter assays in the squid, juvenile aposymbiotic animals were inoculated as described above with wild-type *V. fischeri* containing either the control plasmid pVSV209, or the heme uptake cluster gene reporter plasmids pAKD911 or pAKD913. At various time points post-inoculation, animals were anesthetized using 0.12M $MgCl_2$ and the light organ tissue imaged using a Nikon (Melville, NY) Eclipse E600 epifluorescence microscope equipped with a Nikon 96157 red filter cube, a Nikon 41017 green filter cube, and a Nikon Coolpix 5000 camera for image capture. Similar results were observed with *V. fischeri* harboring pAKD911 and pAKD913, therefore images are only shown for pAKD911-containing *V. fischeri*. At least five animals were analyzed at each time point, with images from one representative animal shown.

In colonization competition experiments, juvenile squid were exposed to mixed inocula with roughly equal numbers of each cell type for 15 h before being rinsed in inoculum-free Instant Ocean. The unmarked deletion mutant AKD910 was competed against AKD100, an erythromycin-resistant mini Tn-*7-ermR* marked derivative of *V. fischeri* wild-type strain ES114. AKD100 has no apparent colonization defects when competed against wild-type *V. fischeri* ES114 (data not shown), and therefore was used as a marked strain with wild-type competitiveness in these assays. Strain ratios were determined after dilution plating by patching to LBS medium containing erythromycin. Competitiveness is presented as a relative competitive index (RCI), which was calculated by dividing the AKD910 to AKD100 ratio in the squid by the AKD910 to AKD100 ratio of the inoculum. A RCI value of 1 indicates the strains competed equally during host colonization, whereas a RCI value of <1 indicates AKD100 (wild type) out-competed the mutant. Data shown for the ~24 and ~96 h time points are combined from two independent experiments, whereas the data shown for the ~48 and ~72 h time points are each from a single experiment.

GFP-based reporter assays in culture

V. fischeri ES114 harboring pAKD911 or pAKD913 was grown overnight in LBS medium containing kanamycin and either with or without 100 μM of the iron chelator 2,2′-bipyridyl at 28°C with shaking. Cultures were diluted 100-fold into 25 ml of the same medium type in

125-ml Erlenmeyer flasks and incubated at 24°C with shaking until the cultures reached an OD_{595} of between 0.93 and 0.99. Ten μl of culture was transferred onto a spot of LBS with 1% agarose on a glass slide and images were taken using 100X objective using the epifluorescence microscope described above. All images used an aperture setting of F4.6, and the exposure time was 0.03 s for bright field and 1.0 s for GFP images. Identical results were obtained in experiments with cultures at varying optical densities (OD_{595} of 0.5 to 1.5), and cultures grown at 28 °C (data not shown).

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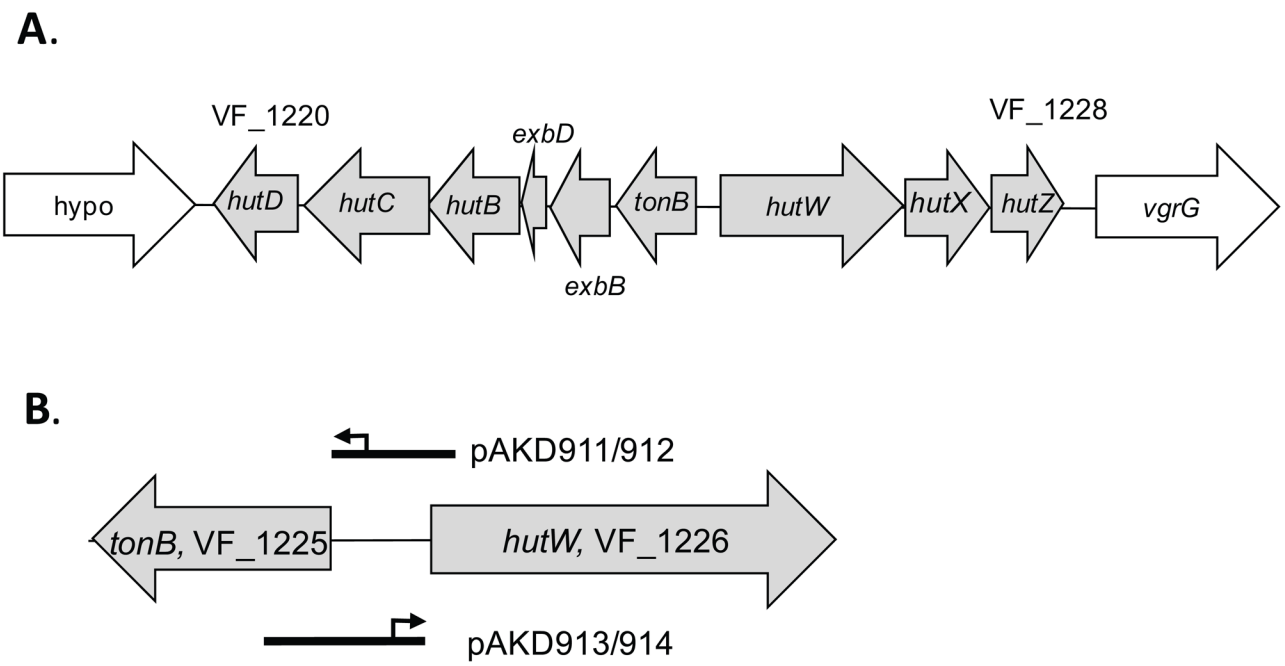


Figure 1.

Organization of the putative heme uptake gene cluster in *V. fischeri* ES114. **A**. The region corresponds to open reading frames VF_1220 to VF_1228 (left to right). Comparisons of these gene products to known heme uptake proteins are described in Table 2. Flanking open reading frames (VF_1219, hypothetical protein and VF_1229, VgrG) are depicted by white arrows. **B**. Representation of the DNA fragments cloned to generate the reporter plasmids. Arrows indicate the approximate region of the putative promoter for each construct.

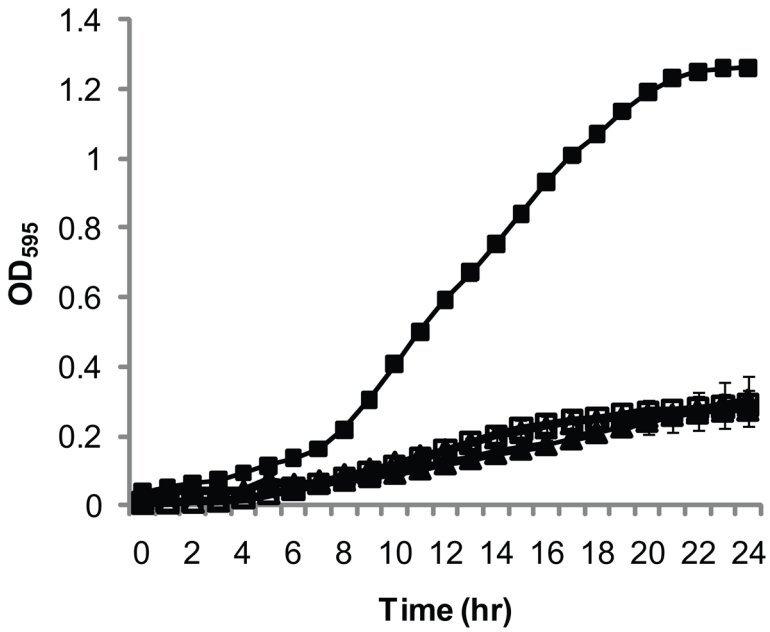


Figure 2.

The putative heme uptake genes are necessary for growth of *V. fischeri* with hemin as the sole iron source. Cultures of wild type and AKD910 (ΔVF_1220-1228) were grown in mineral salts mediumcontaining iron chelator, with and without hemin added. Optical density was measured over time for wild type grown without hemin (open squares), wild type grown with hemin (filled squares), AKD910 grown without hemin (open triangles), and AKD910 grown with hemin (filled triangles). Error bars represent standard error of the mean.

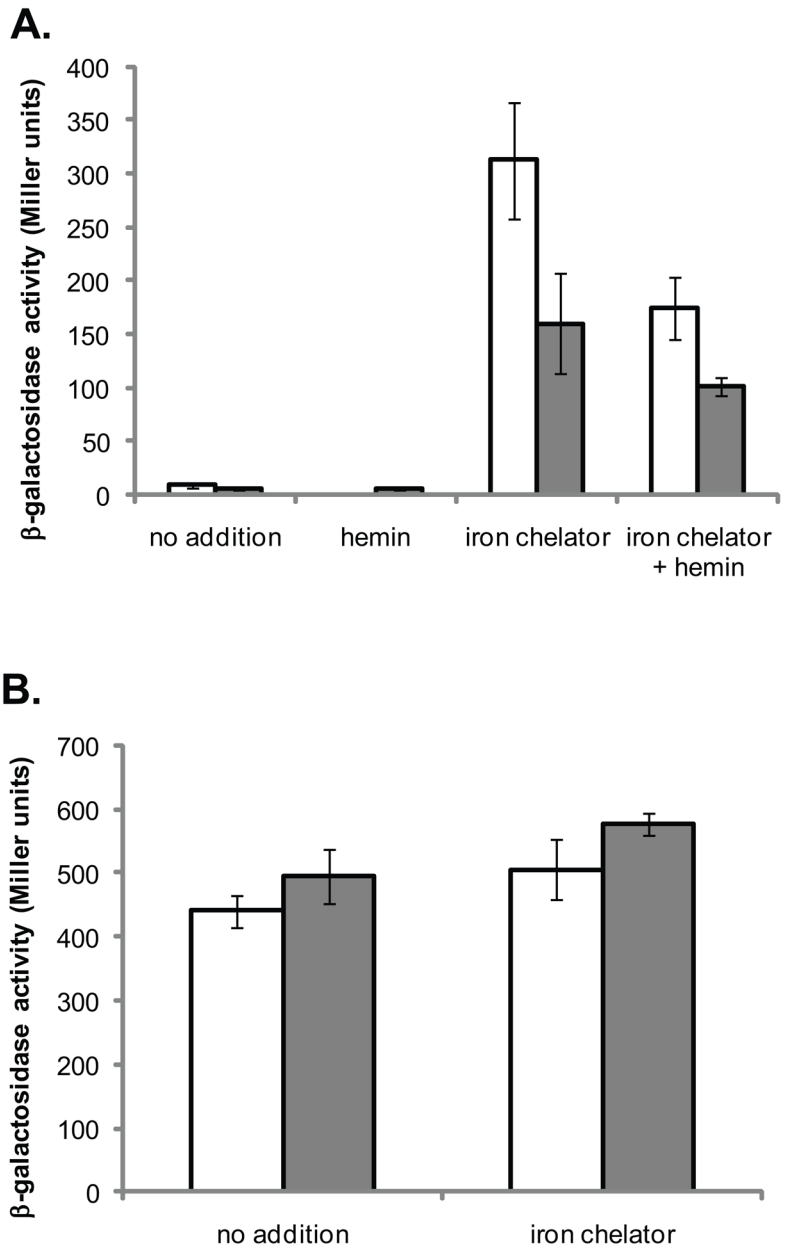


Figure 3.

Heme-uptake promoter activity is induced in response to low iron concentrations and this effect is mediated through Fur. pAKD912 (P_{VF_1225}-*lacZ*) or pAKD914 (P_{VF_1226}-*lacZ*) (see Fig. 1B) was introduced into **A.** wild-type *V. fischeri*, or **B**. a strain lacking *fur* (YLW111). White bars indicate strains containing pAKD912, while shaded bars indicate strains containing pAKD914. Where indicated, iron chelator and/or hemin were added to the culture medium. A student's t-test was used to determine whether the addition of hemin to cultures containing iron chelator significantly influenced the Miller unit values for each reporter in the wild-type background ($p < 0.05$). Although the trend of a decrease in promoter activity in response to hemin was consistent in all experiments, the difference was not consistently significant, and was not significant in the experiment shown.

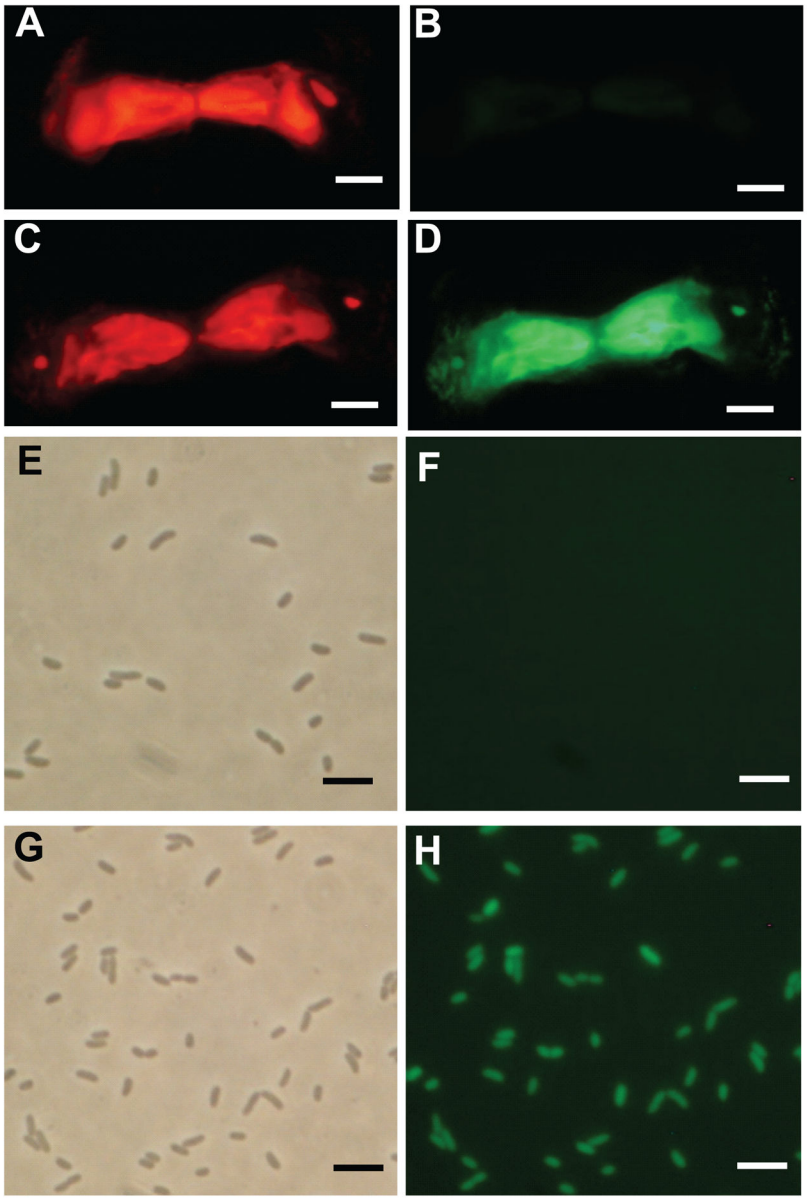
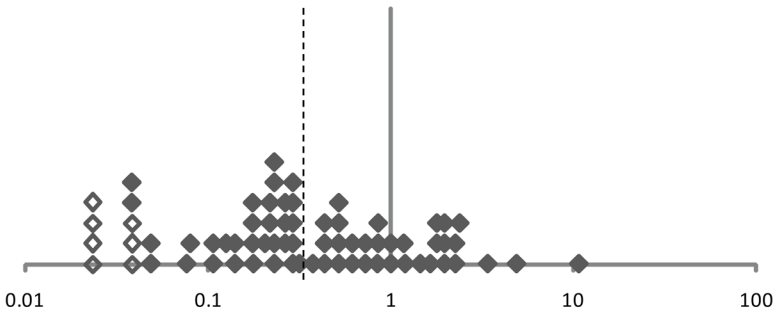


Figure 4.

Heme uptake gene cluster promoter activity is induced during symbiotic colonization, and GFP expression patterns are similar to those observed in culture under low iron conditions. **A–D**. Aposymbiotic juvenile *E. scolopes* were inoculated with either wild-type *V. fischeri* containing the control plasmid pVSV209 where RFP is expressed constitutively and there is no promoter to drive *gfp* expression (A and B), or wild-type *V. fischeri* containing pAKD911, where RFP is expressed constitutively and *gfp* expression is under the control of the promoter associated with the VF_1225-VF_1220 gene cluster (C and D). Epifluorescence images of individual juvenile squid light organs 28 h post-inoculation were taken using either a filter to visualize RFP (A and C) or GFP (B and D). Numbers of bacteria per light organ were determined by plating and, for these images, were 2.76×10^5 and 3.31×10^5 for *V. fischeri* containing pVSV209 and pAKD911, respectively. Similar results were observed at 14 and 48 h post-inoculation (data not shown). **E–H.** Microscopic images of individual *V. fischeri* cells containing pAKD911 either captured using regular light (E and G) or using a filter to visualize GFP (F and H). Cells were grown in LBS medium either without (E and F) or with (G and H) iron chelator. Corresponding to the β-galactosidase reporter assays (Fig. 3), GFP expression is

detectable only when the iron chelator is present in the culture medium. Scale bar indicates approximately 100 μm for A–D and approximately 5 μm for E–H.

A



B

timepoint	number of squid	number of experiments	average RCI	*p*-value
23 , 24h	32	2	1.2	0.67
39 h	19	1	0.46	0.04
72 h	28	1	0.61	0.07
94 ,100 h	70	2	0.33	$8.85\ e^{-10}$

Figure 5.

Heme uptake and/or utilization contributes to symbiotic colonization. **A**. Graphical representation of colonization competitiveness in the combined 94 and 100 h assays. Each symbol represents a RCI value from one animal. The dashed line indicates the average RCI value (0.33) for all animals in the experiment. The animals represented by open symbols were clonally colonized by wild-type *V. fischeri*. **B.** Numerical data for all time points assayed. For the ~ 24 hour and ~ 96 h time points, data was combined from two independent experiments. Significance was calculated using a student's t-test.

Table 1

Strains and plasmids used in this study

Strains or Plasmids	Characteristics	Source
Strains		
Escherichia coli		
DH5α	F′/*endA1 hsdR17 glnV44 thi-1 recA1 gyrA relA1* Δ(*lacIZYA-argF*)*U169deoR*(ϕ80*dlacI*Δ(*lacZ*)*M15*)	(Hanahan, 1983)
DH5αλ*pir*	λ*pir* derivative of DH5α	(Dunn *et al.*, 2005)
Vibrio fischeri		
ES114	wild-type *V. fischeri*	(Boettcher & Ruby, 1990)
AKD100	ES114 with a mini-Tn*7* insertion; Em^R	this study
AKD910	ES114 ΔVF_1220-1228; heme uptake cluster gene deletion mutant (allele exchanged from pAKD910)	this study
YLM111	ES114 Δ*fur* (VF_0810)	this study
Plasmids		
pAKD701	*E. coli-V. fischeri* shuttle vector, RP4 *oriT*, pES213 and R6K replication origins, promoterless *lacZ*, constitutive *rfp*, Kn^R	(Dunn & Stabb, 2008)
pAKD910	ΔVF_1220-1228 allele, R6Kγ and ColE1 replication origins, RP4 *oriT*, Em^R, Kn^R	this study
pAKD911	pVSV209 containing the VF_1225 promoter region	this study
pAKD912	pAKD701 containing the VF_1225 promoter region	this study
pAKD913	pVSV209 containing the VF_1226 promoter region	this study
pAKD914	pAKD701 containing the VF_1226 promoter region	this study
pEVS104	conjugative helper plasmid, R6Kγ replication origin, Kn^R	(Stabb & Ruby, 2002)
pEVS107	pEVS94S derivative, mini-Tn*7*; mob; Em^R Kn^R	(McCann *et al.*, 2003)
pVSV209	*E. coli-V. fischeri* shuttle vector, RP4 *oriT*, pES213 and R6Kγ replication origins, promoterless *gfp* and cm^R, constitutive *rfp*, Kn^R	(Dunn *et al.*, 2006)
pYLW62	Δ*fur* allele, R6Kγ and ColE1 replication origins, RP4 *oriT*, Em^R, Kn^R	this study

Table 2

Comparison of the *Vibrio fischeri* hemin uptake gene cluster to known proteins

***V. fischeri* ORF**	**assignment**	**database comparison**	**% identity**	**% similarity**
VF_1220	HutD (259 aa)	*Vibrio anguillarum*[a], HuvD, ABC transporter ATPase(199 aa), CAF25489, (Mourino *et al.*, 2004)	61% (118/195 aa)	77% (145/195 aa)
VF_1221	HutC (345 aa)	*V. anguillarum*, HuvC, inner membrane permease (314 aa), CAF25488 (Mourino *et al.*, 2004)	74% (230/314 aa)	89% (277/314 aa)
VF_1222	HutB (282 aa)	*V. anguillarum*, HuvB, periplasmic hemin-binding protein, (282 aa), CAF25487 (Mourino *et al.*, 2004)	61% (180/282 aa)	75% (209/282 aa)
VF_1223	ExbD (139 aa)	*V. anguillarum*, ExbD, energy transducer for active transport, (136 aa), CAD43043 (Mourino *et al.*, 2004)	59% (80/136 aa)	76% (103/136 aa)
VF_1224	ExbB (227 aa)	*V. anguillarum*, ExbB, energy transducer for active transport, (235 aa), CAD43042 (Mourino *et al.*, 2004)	59% (120/221 aa)	64% (162/221 aa)
VF_1225	TonB (254 aa)	*V. anguillarum*, TonB, energy transducer for active transport (240 aa), CAD43041 (Mourino *et al.*, 2004)	47% (124/264 aa)	63% (164/264 aa)
VF_1226	HutW (458 aa)	*Vibrio cholerae*[b], HutW, (455 aa), VCA0909 (Wyckoff et al., 2004)	59% (261/443 aa)	77% (338/443 aa)
VF_1227	HutX (178 aa)	V. anguillarum, HuvX, (171 aa), CAD43040 (Mourino *et al.*, 2004)	64% (102/161 aa)	82% (131/161 aa)
VF_1228	HutZ (175 aa)	*V. anguillarum*, HuvZ, (176 aa), CAD43039 (Mourino *et al.*, 2004)	70% (119/172 aa)	84% (143/172 aa)

[a] *V. anguillarum* serovar 01, accession or ORF number and reference provided

[b] HutW is not present in *V. anguillarum*, but is present in *V. cholerae* O1 biovar El Tor strain N16961