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Novel effects of a transposon insertion in the *Vibrio fischeri glnD* gene: defects in iron uptake and symbiotic persistence in addition to nitrogen utilization

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Summary

Vibrio fischeri **is the sole species colonizing the light-emitting organ of the Hawaiian squid, *Euprymna scolopes*. Upon entering the nascent light organ of a newly hatched juvenile squid, the bacteria undergo morphological and physiological changes that include the loss of flagellation and the induction of bioluminescence. These and other events reveal a pattern of genetic regulation that is a response to the colonization of host tissue. In this study, we isolated and characterized a *glnD*::mTn*5*Cm mutant of *V. fischeri*. In addition to the predicted defects in the efficiency of nitrogen utilization, this *glnD* mutant had an unexpected reduction in the ability to produce siderophore and grow under iron-limiting conditions. Although the *glnD* mutant could colonize juvenile squid normally over the first 24 h, it was subsequently unable to persist in the light organ to the usual extent. This persistence phenotype was more severe if the mutant was pregrown under iron-limiting conditions before inoculation, but could be ameliorated by the presence of excess iron. These results indicate that the ability to respond to iron limitation may be an important requirement in the developing symbiosis. Supplying the *glnD* gene *in trans* restored normal efficiency of nitrogen use, iron sequestration and colonization phenotypes to the *glnD*::mTn*5*Cm mutant; thus, there appears to be a genetic and/or metabolic linkage between nitrogen sensing, siderophore synthesis and symbiosis competence in *V. fischeri* that involves the *glnD* gene.**

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Introduction

The maintenance of homeostasis by means of physiological adaptation is an essential cellular process that is tightly mediated by sensory and regulatory proteins whose activities control gene expression and enzymatic activity. While many of these physiological mechanisms are conserved across a wide range of bacterial taxa, unique control circuits have arisen during the adaptation of microorganisms to specific lifestyles. Comparisons of the regulatory cascades present in bacterial species that are adapted to different ecological niches can thus reveal novel regulatory circuits, as well as provide clues to the physiological conditions that characterize their microenvironments.

One distinctive habitat of the luminescent bacterium *Vibrio fischeri* is the light-emitting organ of the Hawaiian sepiolid squid, *Euprymna scolopes* (Ruby, 1996; McFall-Ngai and Ruby, 1998). Newly hatched juvenile squids first acquire their symbionts from the surrounding sea water (Wei and Young, 1989) through a highly specific process; thus, only *V. fischeri* cells are found colonizing the light organ (McFall-Ngai and Ruby, 1991). *V. fischeri* cells enter pores on the surface of the nascent light organ, propelling themselves with their flagella through ciliated ducts and into epithelium-lined crypts (Ruby and Asato, 1993; Graf *et al*., 1994). Within hours after entering these crypts, the bacteria have triggered dramatic developmental changes in the host, including the regression of the light organ's ciliated surface (Doino and McFall-Ngai, 1995; Foster and McFall-Ngai, 1998) and downregulation of the expression of a squid halide peroxidase, an enzyme apparently used by the host to defend against inappropriate bacterial colonization (Weis *et al*., 1996; Small and McFall-Ngai, 1999). By 16 h after inoculation, the symbionts have proliferated to between 10^5 and 10^6 bacteria, lost their flagella and become brightly luminous (Ruby and Asato, 1993). Inside the crypts, these densely packed bacteria are surrounded by a matrix fluid that contains millimolar levels of amino acids in the form of peptides (Graf and Ruby, 1998). Each morning, over 95% of the bacterial culture is expelled from the crypts, and the remaining symbionts repopulate the organ by evening (Lee and Ruby, 1994a; Boettcher *et al*., 1996). Thus, there is a daily cycle of symbiont proliferation, expulsion

and regrowth that is supported by nitrogen-rich nutrients supplied by the surrounding host tissue. While little is known about the nature of nutrient exchange in any bacterial–host association, successful competition for growth in the light organ (Lee and Ruby, 1994b) may include specific metabolic adaptations that allow *V. fischeri* to sense and use this reliably consistent food source efficiently.

In previous studies, we have examined the symbiotic role of specific bacterial genes by evaluating the colonization competence of *V. fischeri* mutants generated by either transposon mutagenesis or allelic exchange (Graf *et al*., 1994; Visick and Ruby, 1996; 1998; Graf and Ruby, 1998). The results of this work have identified nutritional and enzymatic traits that allow *V. fischeri* to initiate a colonization and accommodate to the conditions of the light organ environment. For many host tissue-associated microorganisms, including the pathogenic *Vibrio* species, *V. anguillarum*, which infects fishes, and *V. cholerae*, the causative agent of human cholera, a mechanism for obtaining iron from the host is a colonization requirement (Singer *et al*., 1991; Henderson and Payne, 1994; Litwin and Calderwood, 1994). Animals generally maintain a low concentration of free iron in their tissues, sequestering it with binding proteins such as transferrin. In this way, they protect themselves both from reactive oxygen species that can arise spontaneously in the presence of free iron, as well as from unwanted bacterial infections (Braun and Killman, 1999). Certain bacteria, in turn, produce iron-binding siderophores that have higher affinities than the host's iron-sequestering proteins. These siderophores are often small, cyclic peptides that are secreted extracellularly where they bind ambient iron. The resulting complex is recognized by a specific bacterial surface receptor and transported into the cytoplasm (Moeck and Coulton, 1998).

V. anguillarum responds to low-iron environments by producing the siderophore anguibactin, which is encoded on its virulence plasmid (Crosa, 1997). The synthesis of this molecule is regulated at a number of levels by a complex series of mechanisms that includes a repressor, Fur, antisense RNA and the positive regulator AngR, a leucine-zipper protein (Crosa, 1997). Presumably, having several levels of control minimizes the energetic expense associated with unnecessary siderophore secretion. Less is known about the synthesis of siderophores by other *Vibrio* species (Trick, 1989), except that several molecules other than anguibactin have been reported, including vibriobactin (Wyckoff *et al*., 1999) and the hydroxamate-type siderophore, aerobactin (Haygood and Holt, 1993; Murakami *et al*., 1998). One strain of *V. fischeri* has been reported to secrete aerobactin; however, to our knowledge, no information concerning the regulation of its synthesis or whether other siderophores are produced has been published. In fact, although siderophores have been described in over 30 species of bacterial pathogens, almost nothing is known about iron sequestration and its regulation by species of beneficial bacteria such as *V. fischeri* during their symbiotic colonization of animal tissue.

To investigate whether siderophore production is required for, and perhaps contributes to, the specificity of *V. fischeri* colonization of *E. scolopes*, we have isolated and characterized a bacterial transposon mutant that has a siderophore production defect. Analyses of such a mutant revealed that it carries a transposon insertion in the *V. fischeri glnD* gene, which not only affects the regulation of nitrogen metabolism in this bacterium, but also produces a defect in the ability of the mutant to persist normally in the light organ symbiosis.

Results

Isolation of a V. fischeri *iron sequestration mutant*

We evaluated the ability of transposon mutants of *V. fischeri* to sequester iron on the indicator medium chrome azurol S (CAS) agar. Bright orange haloes surrounding areas of cell growth indicated that the light organ isolate ES114, as well as its derivative ESR1 (Table 1), produced siderophores that sequestered iron from the medium (data not shown). Among 1200 transposon mutants of strain ESR1 screened on CAS agar, two exhibited a potential iron sequestration defect. One mutant produced a smaller orange halo than the parent strain, and a second (SP301) produced no detectable halo. These strains were designated presumptive siderophore production (SP) mutants. Strain SP301 carried a single insertion, as determined by Southern hybridization using the Cm^R gene of the transposon as a probe (data not shown).

Effect of iron chelators on the growth of strain SP301

In a rich nutrient medium (SWT) containing an excess of available iron, the growth rates of SP301 and ESR1 were indistinguishable (data not shown). However, the growth rate of SP301 in a low-Fe medium (IM-5) was less than that of ESR1, and was reduced even further by the addition of 10 μM EDDHA (Fig. 1). In contrast, the addition of 10 μM EDDHA had practically no effect on the growth rate of ESR1. Similarly, in the presence of 50 μM EDDHA, the growth yield of SP301 was reduced by 85%, whereas that of ESR1 was only 20% lower (data not shown). The effect of EDDHA was apparently caused specifically by its ability to sequester iron, because the addition of excess $FeCl_3$ restored the growth rate of SP301 to that observed in the absence of the chelator (Fig. 1). Interestingly, the growth rate of strain SP301 in

Table 1. Bacterial strains, plasmids and phage used in this study.

Designation	Relevant characteristics	Reference or source
Escherichia coli strain		
XL1-Blue	Cloning strain (*recA1*, Tn*10*, Tc^R, *lacI*^{q})	Stratagene
Vibrio fischeri strains		
ES114	Light organ isolate from *Euprymna scolopes*	Boettcher and Ruby (1990)
ESR1	ES114 derivative, spontaneous Rf^R	Graf *et al.* (1994)
SP301	ESR1 *glnD1* (*glnD*::mTn*5*Cm); Rf^R, Cm^R	This study
G11	ES114 transductant, *glnD1*; Cm^R	This study
Plasmids		
pUTmTn5r°Cm	R6K ori, *pir*$^-$, *mob*, mTn*5*Cm	de Lorenzo and Timmis (1994)
pBSII	High-copy cloning vector; Ap^R	Stratagene
pVO8	*V. fischeri* cloning vector; *mob* site; Cm^R, Em^R	Visick and Ruby (1997)
pJG15	pBSII with a 5 kb *Bgl*II fragment carrying mTn*5*Cm and DNA downstream of the SP301 insertion; Ap^R, Cm^R	This study
pJG16	pJG15 with the mTn*5*Cm cassette removed; Ap^R	This study
pJG26	pBSII with an 8.8 kb *Xba*I–*Not*I fragment carrying mTn*5*Cm and DNA upstream of the G11 insertion; Ap^R, Cm^R	This study
pJG28	pBSII with a 2 kb *Eco*RI fragment of the *glnD* gene carried in pJG26; Ap^R	This study
pJG29	pBSII with a 1.5 kb *Hin*dIII fragment of pJG26; Ap^R	This study
pJG40	pVO8 with a 2.8 kb *Eco*RI–*Bgl*II fragment carrying the complete *V. fischeri glnD* from ESR1; Em^R	This study
Phage		
rp-1	*V. fischeri* generalized transducing phage	Levisohn *et al.* (1987)

Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Em^R, erythromycin resistance;Rf^R, rifampicin resistance.

IM-5 medium, even with the addition of excess iron, was not the same as that of its parent. This result suggested that the reduced level of another nutrient(s) in this relatively minimal medium (compared with SWT) resulted in the appearance of a second, non-Fe-related growth defect in SP301. Thus, we next examined the basis of the transposon-induced defect in SP301 at the genetic level.

DNA sequence interrupted by the transposon insertion

The site of the transposon insertion in strain SP301 was determined by cloning the antibiotic resistance gene of mTn*5*Cm along with flanking chromosomal DNA. Southern analysis revealed a 5 kb *Blg*II fragment and an 8.8 kb *Not*I–*Xba*I fragment that hybridized with the Cm^R gene of mTn*5*Cm (data not shown). Between 1 and 3 kb of DNA on either side of the transposon insertion was sequenced (Fig. 2), revealing a large open reading frame (ORF) of 2619 bp with the insert located after nucleotide 1908.

Alignment of 873 residues of the deduced amino acid sequence of this ORF against known protein sequences revealed highly significant similarities (P-value $< 10^{-200}$) to the *glnD* genes of *Escherichia coli* and *Klebsiella pneumoniae*, as well as to the *glnD* homologue *nfrX* of *Azotobacter vinelandii.* Overall, the identities were 52%, 52% and 41% respectively. Regions of identity were observed along the entire length of the deduced protein, although the amino-termini of the proteins were the most similar. This significant degree of amino acid identity throughout the protein indicated that the transposon in strain SP301 is inserted into a *glnD* homologue of *V. fischeri*.

Partial DNA sequences of three ORFs located upstream of the *glnD*-like gene showed derived amino acid sequences that were between 56% and 75% similar to proteins encoded by the *E. coli* genes *map*, *rpsB* and *tsf* (Fig. 2). The relative positions and orientations of these three gene homologues in the *V. fischeri* chromosome are

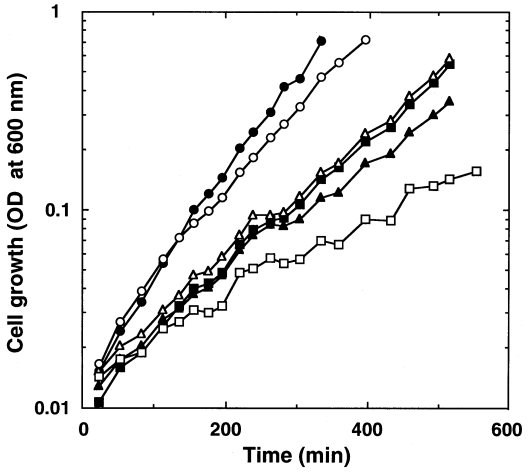

Fig. 1. The effect of iron availability on the growth rate of the *V. fischeri glnD* mutant (SP301) and its parent (ESR1). SP301 growth rates were measured in the low-iron medium IM containing additions of the iron chelator EDDHA to a concentration of 0 (closed square), 5 (closed triangle) or 10 (open square) μM. In one case, the IM medium was supplemented with both 10 μM EDDHA and 25 μM $FeCl_3$ (open triangle). Growth rates were also measured for strain ESR1 in IM medium that contained EDDHA at a concentration of either 0 (closed circle) or 10 (open circle) μM.

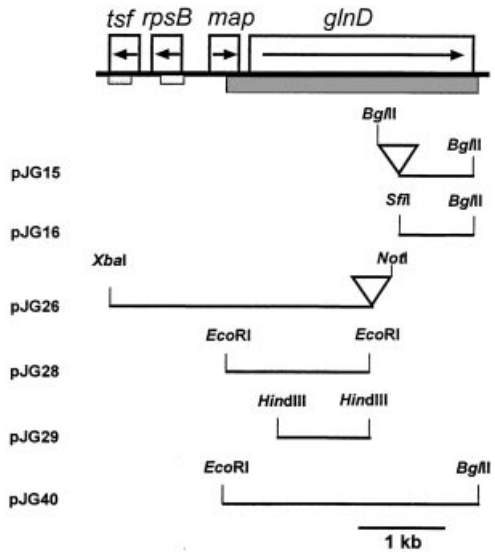

Fig. 2. Genetic map of the region around the *V. fischeri glnD* gene. Upstream of the *glnD* gene are apparent homologues of the *E. coli tsf*, *rpsB* and *map* genes, as determined by sequencing the squared-off areas under the bold line of the map. Plasmids used to clone fragments of this region are identified below the map. The presence of the mTn*5*Cm sequence (approximately 3.5 kb) in some of the fragments is indicated by an inverted triangle. The fragment in pJG40 was cloned from strain ES114.

also identical to those of their counterparts in *E. coli* (van Heeswijk *et al*., 1993).

Linkage of the transposon insertion to the siderophore phenotype

The *glnD* gene of *E. coli* plays a well-characterized role in sensing the nitrogen status of the cell; however, to our knowledge, no effect on siderophore regulation has been reported. Because such a linkage appeared to exist in *V. fischeri* SP301, we examined whether the transposon and the iron phenotype were linked directly. To accomplish this, the transposon-encoded Cm^R gene was transduced into *V. fischeri* ES114 to recreate the *glnD* mutation in the wild-type genetic background. A total of six Cm^R transductants was isolated, and all these produced no detectable siderophores (i.e. no halo was formed on CAS agar plates). In addition, like strain SP301, the transductants also produced small (relative to wild type) colonies on CAS agar plates. Taken together, these results suggested that the *V. fischeri glnD* gene and the synthesis of at least one siderophore are genetically linked in this bacterium.

Utilization of nitrogen and carbon sources

The identification of *glnD* as the disrupted locus in SP301 suggested that this strain's reduced growth rate in IM-5 medium relative to that of its parent (Fig. 1) might be linked to the reduced availability of nitrogen compounds in IM-5. Thus, we compared the utilization of nitrogen sources by strain G11, one of the transduced *V. fischeri glnD* mutants, with that of its parent, strain ES114. Both strains were pregrown in a minimal medium containing NH_4Cl as the sole nitrogen source, and either glycerol (MM-gly) or ribose (MM-rib) as the carbon source. The resulting cells were then inoculated into one of three media: SWT, MM-gly or MM-rib; in the last two, the NH_4Cl was replaced by various organic nitrogen sources. The growth rates of these cultures were then monitored, and the generation times were calculated. In the nutrient-rich SWT medium, no difference in the growth rates between G11 and ES114 was observed (Table 2). However, in the minimal media containing NH_4Cl, the growth rate of G11 was 37–52% lower than that of ES114, independent of the carbon source (Table 2). The rate of growth of G11 was also lower than that of ES114 in all the MM-gly media containing organic nitrogen compounds. The most dramatic difference in the growth of these strains was observed with cAMP as the nitrogen source: in this medium, the generation time of strain G11 was 3.0–3.4 times greater (Table 2).

The strains showed much smaller differences in growth rates (20–50%) when grown on either glutamate or glutamine (Table 2). Interestingly, while glutamate was one of the poorest sources of nitrogen for growth of the wild-type strain, it supported one of the fastest relative growth rates of strain G11. For most of the nitrogen sources tested, G11 also had a longer lag phase than ES114 (data not shown). Finally, with the exception of cAMP, no differences in final growth yield were observed when the measurements were extended to 48 h (data not shown). These data demonstrate that a defect in *glnD* results in a reduced ability of *V. fischeri* cells to use all the nitrogen sources tested effectively.

Cloning of the V. fischeri glnD*-like gene*

Southern analysis of total DNA from ES114, probed with a 1.5 kb *Hin*dIII fragment (pJG29) of pJG26 that encodes an internal region of the *glnD* homologue (Fig. 2), revealed a single hybridizing 7 kb *Eco*RI fragment (data not shown), suggesting that only one *glnD*-like gene was present in *V. fischeri*. A size-fractionated *Eco*RI library of ES114 DNA carried in *E. coli* was screened by colony hybridization using the 1.5 kb *Hin*dIII probe. Two positive clones were identified, and the *V. fischeri* DNA carried in one of these was subcloned into pVO8 as an *Eco*RI–*Bgl*II fragment, yielding pJG40 (Table 1).

Complementation of the iron defect in strain G11 by the V. fischeri glnD *gene*

Plasmid pJG40 carrying the complete *V. fischeri glnD*-like

Table 2. Generation times of strains G11 and ES114 using different carbon and nitrogen sources.

Source of [b]		Generation time (h)[a]			
		Pregrown on glycerol ± NH_4		Pregrown on ribose ± NH_4	
Carbon	Nitrogen	G11	ES114	G11	ES114
Glycerol	NH_4Cl	5.6	2.7	4.9	3.0
Glycerol	Arginine	6.9	3.4	5.0	3.3
Glycerol	Asparagine	ND[c]	ND	7.6	5.5
Glycerol	cAMP	9.2	2.7	11.6	3.8
Glycerol	Glutamate	5.6	4.3	5.8	4.6
Glycerol	Glutamine	4.7	3.2	4.0	3.2
Glycerol	Serine	6.2	2.5	7.0	4.4
Ribose	NH_4Cl	6.2	2.3	6.1	3.0

a. Generation time (in hours) $= [t_1 - t_0]\ [\ln OD_1 - \ln OD_0]^{-1}$, where OD_0 is the optical density of the culture at time zero (t_0), and OD_1 is the optical density at a later time (t_1). The generation times in a rich medium (SWT) were 1.5 for ESR1 and 1.5 for SP301, regardless of their pregrowth conditions.
b. Nutrient concentrations were: glycerol (40 mM); ribose (24 mM); nitrogen sources (10 mM).
c. Not determined.

gene, as well as the vector control pVO8, was electroporated into either the wild-type ES114 or the *glnD* mutant G11. The resulting strains were then observed for their ability to synthesize siderophores on CAS agar (Fig. 3). While G11 (pVO8) produced no halo, G11 (pJG40) produced a bright orange halo. Because pJG40, which contained essentially only the predicted *glnD* ORF (there are only 325 bp upstream of the start codon and 35 bp of downstream sequence; Fig. 2), was able to complement the siderophore phenotype, the presence of an intact *glnD* gene appears to be sufficient to restore normal siderophore synthesis and function.

Similarly, the *glnD* gene could also restore wild-type growth characteristics to strain G11 cultured in a Fe-limited medium. For example, the growth yield of G11 in IM-5 medium containing the iron chelator EDDHA was significantly lower than that of the wild type (Fig. 4); however, this defect was fully complemented by the presence of a copy of the intact *V. fischeri glnD* gene on pJG40.

Complementation of the nitrogen defect in strain G11 by the V. fischeri glnD *gene*

Strain G11 exhibited a diminished growth rate (Table 2) and growth yield (Fig. 5) when cultured in a minimal medium containing single sources of nitrogen. The ability of pJG40 to restore normal cell yields was examined during growth on three nitrogen sources: ammonium, arginine and cAMP. The results indicated that the presence of the *glnD* gene was effective in each case (Fig. 5). Taken together with the results described above, these data confirm that, in *V. fischeri*, the iron and nitrogen phenotypes are both regulated by the product of the *glnD* gene.

Colonization of juvenile squid

Both the *V. fischeri glnD* mutant and its parent strain were able to colonize the nascent light organs of juvenile squid to an equivalent extent during the first 24 h; however, at subsequent times, the *glnD* mutant showed a colonization defect. While the level of colonization by the parent strain at 40 h and 72 h remained essentially constant (i.e. there was less than a 10% decrease), in light organs colonized

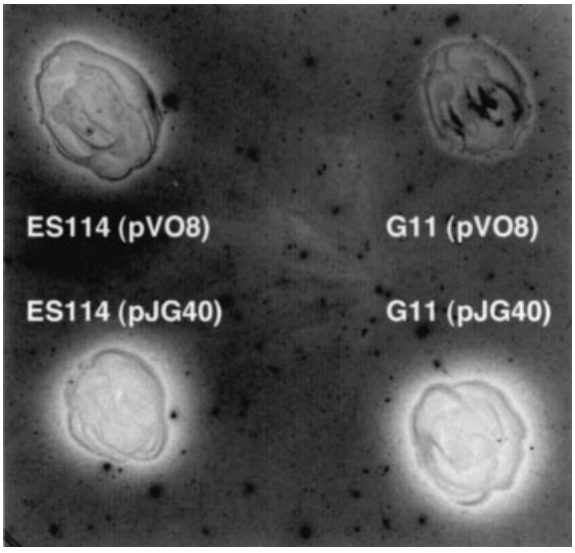

Fig. 3. Characterization of siderophore production by strains of *V. fischeri*. Four strains were grown on blue CAS agar, which turns a bright orange (a light grey halo in this photo) when iron is removed from the CAS dye by the action of an excreted siderophore(s). Strain ES114 is the wild-type siderophore-producing parent of strain G11, the *glnD*::mTn*5*Cm null mutant defective in siderophore synthesis, leading to a much-diminished halo. This defect is complemented by pJG40, which carries the wild-type *glnD* gene *in trans*, but not by pVO8, the vector control.

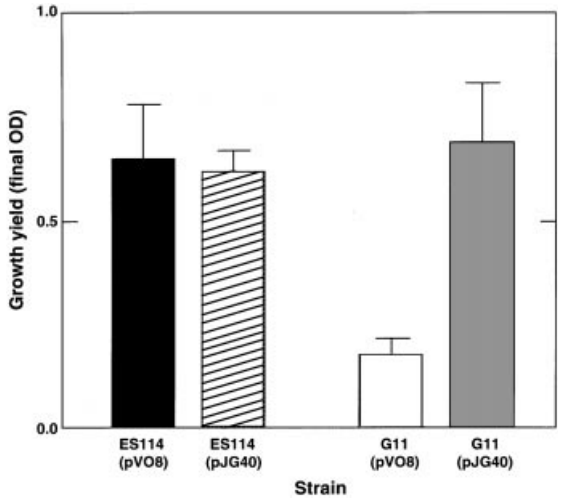

Fig. 4. Growth yields of *V. fischeri* strains cultured in iron-limited media. Wild-type (ES114) and *glnD* mutant (G11) strains, carrying either an intact copy of *glnD* (pJG40) or the vector alone (pVO8), were grown in IM-5 medium containing 25 μM EDDHA. The maximum culture OD was achieved within 40 h of inoculation. The significantly reduced yield of strain G11 was restored to wild-type levels by the complementing copy of *glnD* on pJG40. Error bars indicate the SEM of triplicate cultures. A similar pattern was seen when these four strains were grown in IM-5 containing 50 or 100 μM EDDHA.

by the *glnD* mutant, the average abundance of the symbionts had dropped by 55% and 85% at 40 h and 72 h respectively. This persistence defect was not observed if the colonization experiments were performed in sea water containing an excess (20 μM total) of free iron: up to at least 6 days after infection, there was no significant difference between the average number of bacteria present in light organs colonized by the parent or by the mutant (data not shown).

Many bacteria store iron in their cytoplasm using proteins called ferritins, which allow continued, albeit temporary, growth in the absence of an external iron source (Grossman *et al.*, 1992). Thus, we reasoned that, if the reduced capacity to obtain iron effectively from the host tissues was an important defect in the *glnD* mutant, then the extent of iron starvation experienced by *V. fischeri* cells before inoculation might emphasize the inability to persist. Therefore, juvenile squid were exposed to parent or *glnD* mutant bacteria that had been pregrown in IM medium under iron limitation for either 9 h or 42 h before inoculation. Forty-eight hours after inoculation, cells of the *glnD* mutant had colonized the light organ to a significantly lower extent than had similarly iron-limited cells of the parent strain (Fig. 6). These results all suggested that the *glnD* mutant had a reduced capacity to persist in the light organ as a result of an inability to obtain sufficient iron there.

Complementation of the symbiotic defect in strain G11 by the V. fischeri glnD *gene*

When compared with wild type, strain G11 (like SP301) exhibited a significantly lower level of colonization of squid light organs after 39 h, as judged by both the level of luminescence produced by the squid and the number of *V. fischeri* colony-forming units (cfu) present in light organ homogenates (Table 3). When G11's defective genomic copy of *glnD* was complemented by the intact *glnD* gene carried on pJG40, these symbiotic phenotypes were restored.

Transposon stability and symbiont specificity in the colonized light organ

To ensure that no reversions of the iron sequestration phenotype had occurred as a result of selection during colonization, *V. fischeri* cells were isolated from *glnD* mutant-colonized light organs after 72 h and tested on CAS plates for evidence of siderophore production. All 15

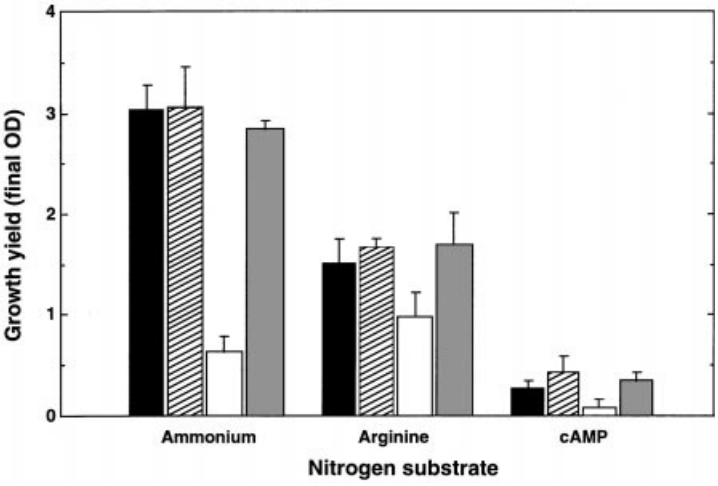

Fig. 5. Growth yields of *V. fischeri* strains cultured in minimal media containing single nitrogen sources. The relative growth yield (determined by the maximum culture OD after 24 h) was compared between strain ES114, carrying either the vector alone (solid bar) or an intact copy of *glnD* (hatched bar), and strain G11, carrying either the vector alone (open bar) or an intact copy of *glnD* (shaded bar). Cells were grown in MM-rib medium containing ammonium, arginine or cAMP. Error bars indicate the SEM of triplicate cultures.

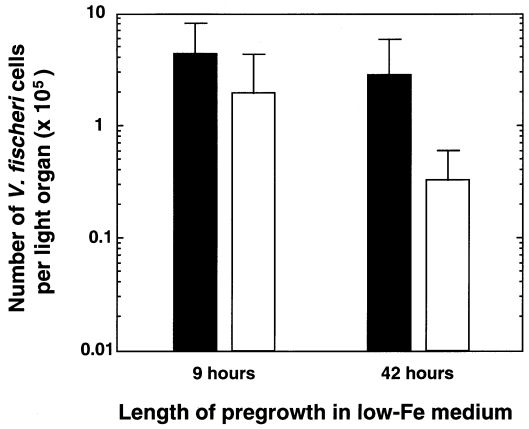

Fig. 6. The effect of the iron limitation during pregrowth of the *V. fischeri glnD* mutant, SP301 (hatched bars), and its parent, ESR1 (solid bars), on their ability to persist in the juvenile squid light organ. The strains were pregrown in the iron-limited medium IM for either 9 h or 42 h, creating a condition of low or moderate iron insufficiency respectively. Each of these four cell cultures was used to infect 10 juvenile squid, and the average numbers of *V. fischeri* cfus present in their light organs after 48 h were determined. Error bars represent 1 SD.

bacterial isolates tested had retained the defect in iron sequestration. Chromosomal DNA from six of these isolates was examined further by Southern hybridization using a transposon sequence as the probe. All six showed the same hybridization pattern as the inoculating mutant strain, demonstrating the stability of the mTn*5*Cm insertion throughout the course of the colonization experiments.

To determine whether the ability of *V. fischeri* cells to compete for iron in the light organ environment might play an important role in their ability to exclude other marine bacteria from co-colonizing the squid, homogenates of light organs colonized by the *glnD* mutant for 72 h were spread onto a rich sea water medium (SWT) that allows the growth of most culturable marine bacteria. After incubation at room temperature for 48 h, these plates were examined for the presence of colonies that were not recognizable as *V. fischeri*. The distinct morphology of *V. fischeri* colonies (pale yellow colour, shiny and 2–3 mm in diameter) allowed the detection of presumptive contaminant species; however, in all cases, the presence of colonies that were not *V. fischeri* in origin was below the level of detection (< 14 cfu per organ). These results suggest that a reduced abundance of free iron in the light organ, which we hypothesize is normally created by *V. fischeri* siderophore production, does not play a necessary or significant role in the maintenance of the specificity of this association.

Discussion

In this study, we characterized a strain of *V. fischeri* that had lost the ability to sequester iron normally as a result of a single transposon insertion in its *glnD* homologue. The resultant iron sequestration phenotype was further linked to the *glnD* insertion in two ways: (i) co-transduction of the transposon insertion and the iron phenotype; and (ii) the restoration of the wild-type phenotype when an intact copy of *glnD* was supplied to the mutant *in trans*. In addition to growing poorly under iron-limiting conditions, the *glnD* mutant was inefficient in using nitrogen sources and was unable to persist normally in the light organ of *E. scolopes*, the natural symbiotic host of these bacteria. Thus, the product of the *glnD* gene is involved, either directly or indirectly, in the control of more than one physiological function in *V. fischeri*. Co-regulation of multiple functions such as these may play a role in the bacterium's adaptation to life as a light organ symbiont.

The *glnD* gene plays an integral role in the response of *E. coli* and other enteric bacteria to changes in the availability of nutritional nitrogen, which is first detected through the relative cytoplasmic abundances of glutamine and 2-oxoglutarate (reviewed by Magasanik, 1996). This ratio is sensed by the bifunctional uridylyl-removing enzyme/uridylyltransferase (UR/UTase) encoded by *glnD*. UR/UTase ultimately regulates both the activity of the key nitrogen-assimilating enzyme glutamine synthetase (GlnA), as well as the transcriptional activation of *glnA* and other nitrogen level-controlled genes through NtrBC and the alternative sigma factor, σ^{54} (Kustu *et al.*, 1979). UR/UTase responds to a high ratio of glutamine to 2-oxoglutarate by removing the uridylyl groups on the P_{II} protein. De-uridylylated P_{II} acts together with NtrB to deactivate the transcriptional regulator NtrC, which results in a diminished expression of *glnA* and other genes in the

Table 3. Complementation of symbiosis defects in a *glnD* mutant.

Strain	Luminescence in host (quanta s^{-1} $animal^{-1}$ $\times$ 10^5)[a]	Colonization level (cfu light $organ^{-1}$)[a]
ES114 (pVO8)	340 (± 45)	220 000 (± 25 000)
G11 (pVO8)	75 (± 50)	52 000 (± 17 000)
G11 (pJG40)	220 (± 55)	160 000 (± 20 000)
G11 (pVO8) + $FeCl_3$[b]	290 (± 120)	ND[c]

a. Average of five animals sampled at 39 h after infection (± SEM).
b. $FeCl_3$ was added to the sea water to a final concentration of 30 μM.
c. Not determined.

σ^{54} regulon (Magasanik, 1996). In addition, P_{II} inhibits the activity of GlnA directly by increasing its degree of adenylation (Reitzer and Magasanik, 1987). Conversely, under nitrogen-limiting conditions, the transcription of σ^{54}-regulated genes is enhanced, and glutamate synthetase activity increases. This complex cascade system provides a controlled and responsive system for sensing the availability of nitrogen to the growing cell. The physiological mechanisms by which symbiotic bacteria metabolically adapt to the nitrogen and carbon sources that they encounter while in their hosts are likely to be similarly well regulated; however, with the exception of N_2-fixing symbionts of legumes (O'Connell *et al*., 1998) and *Azolla* (Madan and Nierzwicki-Bauer, 1993; Meeks, 1998), little has been described about the details of this regulation.

Animals and plants typically maintain specific populations of one or more species of microorganisms in co-operative associations on and within their tissues (Handelsman and Stabb, 1996; McFall-Ngai, 1998). To be successful colonists, microbial symbionts must accommodate to the specific conditions present in host tissues. Among the most important of these conditions are the kinds and amounts of host-derived nutrients that are available, especially if there is competition for these nutrients among different species, or even genetic lineages of the same species. Thus, characteristic, but distinct, assemblages of benign microorganisms have evolved that dominate such diverse niches as the oral cavity, the urogenital tract and the skin (Hentges, 1993; Whittaker *et al*., 1996). Presumably as a result of their radically different diets, animals as diverse as termites, cows and humans have distinct populations of microbiota in their enteric tracts (Breznak and Brune, 1994; Flint, 1997). For example, the presence of *E. coli* as a major facultative anaerobe in the large intestine of most mammals is believed to result in part from this species' ability to use the nutrients routinely encountered there effectively (Ochman and Wilson, 1987), perhaps including specific sources of organic nitrogen, a process that is controlled in laboratory culture in part by the *glnD* gene product (Magasanik, 1996). Unfortunately, the inability to separate the activities and interactions of each of the hundreds of species present in the enteric tract of mammals or other animals has, until recently (Hooper *et al*., 1998), confounded experimental studies of the role of regulators such as *glnD* in their natural environment.

Because host tissues are maintained in a state of remarkable homeostasis, a single environmental cue can be used to signal a number of distinct responses simultaneously in a colonizing bacterium. These co-ordinately regulated responses of a gene family, or regulon, to an environmental signal are found among pathogenic bacteria (Mekalanos, 1992) and often act through single sensor molecules that can modulate the expression of a group of otherwise unrelated physiological activities (Neidhardt and Savageau, 1996). One nutritional condition encountered by *V. fischeri* cells during their colonization of the squid light organ crypts is the presence of an abundance and variety of host-derived amino acids and peptides (Graf and Ruby, 1998). Like enteric bacteria, *V. fischeri* apparently co-ordinates N metabolism through the activity of the product of the *glnD* gene, which, if defective, leads to less efficient growth on amino acids (Table 2). However, unlike *E. coli* (Bloom *et al*., 1978), this defect of the *glnD* mutant is not relieved by the addition of glutamine in a minimal medium (Table 2). Nonetheless, a mixture of nitrogen sources such as that present in a tryptone-based medium does allow the *V. fischeri glnD* mutant to assume a wild-type growth rate. Interestingly, a *glnD* mutation also results in an inability of *V. fischeri* to respond effectively to iron limitation conditions (Fig. 3), resulting in the loss of the ability of the mutant to persist in the light organ association (Fig. 6, Table 3).

In this study, we have demonstrated that the *V. fischeri glnD* gene is a symbiotic determinant, required for persistence in the co-operative light organ association with *E. scolopes*. We propose that the defect in iron acquisition (rather than nitrogen metabolism) may play a dominant role because of two observations: (i) the severity of the symbiosis defect depended upon the degree to which the bacterial inoculum was starved of iron before colonization; and (ii) the defect was alleviated in the presence of excess iron. The loss of an ability to sequester iron has also been reported to result in a defect in tissue colonization by a number of species of pathogenic bacteria (Wooldridge and Williams, 1993). For instance, mutants of *V. anguillarum* that are no longer able to produce siderophores exhibited a 10^5-fold increase in the LD_{50} (Crosa, 1997). Perhaps a siderophore null mutant of *V. fischeri* would show a more dramatic symbiosis phenotype as well.

In some bacteria, homologues of *glnD* have been found to regulate other metabolic functions, such as nitrogen fixation (Kennedy *et al*., 1994), and σ^{54} can be involved in the expression of structures that are not involved in nitrogen metabolism, such as flagella and pili. Similarly, it was briefly believed that there was a transcriptional link between nitrogen metabolism and iron sequestration in *V. anguillarum* (O'Toole *et al*., 1996), although that link was eventually proved erroneous (O'Toole *et al*., 1997). Thus, there is no previous report of a connection between *glnD* function and siderophore metabolism in any bacterium. However, in view of the data presented in this paper, two possible mechanisms can be postulated. First, the loss of UR/UTase activity in the *V. fischeri glnD* mutant might effect iron sequestration directly by limiting a metabolic intermediate required for the synthesis of the siderophore or by effecting the transcription of a siderophore synthesis

(and/or siderophore receptor) gene(s). Because induction of some siderophore transporters requires the presence of the siderophore (van Heeswijk *et al*., 1993), interference with siderophore synthesis may downregulate both components. Alternatively, the mutation might indirectly diminish siderophore function simply by changing some aspect of the bacterium's general physiology, such as acid production (Rae *et al*., 1997) or inorganic nutrient storage (Grossman *et al*., 1992; Ault-Riche *et al*., 1998).

The fact that *glnD* null mutations in other bacterial species can have results as diverse as the suppression of a cell division mutation in *E. coli* (Powell and Court, 1998) or inhibition of plant chlorosis by *Rhizobium tropici* (O'Connell *et al*., 1998) suggests that the GlnD protein has been recruited, in as yet unknown ways, into controlling different sets of physiological functions in different bacterial species. Some of the effects of the *glnD* mutation in *V. fischeri* are similar to its effect in enteric bacteria, i.e. a decreased rate of growth in minimal media containing various nitrogen sources. In contrast, several other previously reported responses appear to be so species specific that two bacteria as closely related as *E. coli* and *Salmonella enteritidis* do not react similarly (Magasanik, 1996). Thus, it should not be surprising that the *V. fischeri glnD* mutant responds differently to nitrogen-supplemented minimal media than does *E. coli*. Interestingly, while glutamine does not fully restore a wild-type growth rate to the *V. fischeri glnD* mutant, it did support the fastest relative growth rate by this strain (Table 2). While the mechanisms that underlie the details of these responses are as yet unknown, the data presented here support the conclusion that, in *V. fischeri*, as in other described species, GlnD is required for efficient nitrogen utilization.

Perhaps these differences result from distinctive nutrient conditions encountered in the two niches that these two enteric species have become adapted to: the digestive tracts of mammals and of lower vertebrates respectively (Minette, 1984; Ochman and Wilson, 1987). In the case of the *V. fischeri glnD* mutant, there is both an expected nitrogen utilization phenotype and an unexpected iron acquisition phenotype. This novel combination of effects leads us to hypothesize that *glnD* plays an unexpected regulatory or metabolic role in *V. fischeri*, and it may indicate an adaptation by this bacterium to a specialized environment, the squid light organ, that appears to be both rich in amino acids and low in available iron.

Experimental procedures

Bacterial strains and growth conditions

The characteristics and sources of the bacterial strains, plasmids and phage used in this study are listed in Table 1. Generally, *V. fischeri* strains were grown at 28°C in either LBS medium (Graf *et al*., 1994) or SWT medium (Boettcher and Ruby, 1990), solidified with 1.5% (w/v) bacto-agar (Difco Laboratories) as appropriate. *E. coli* cells were grown at 37°C in LB medium (Sambrook *et al*., 1989). Phage transductions were performed using the rich sea water-based medium SWC (Nealson, 1978). When appropriate, chloramphenicol (Cm; for *V. fischeri* 1 μg ml^{-1} and, for *E. coli*, 30 μg ml^{-1}), erythromycin (Em; 5 μg ml^{-1}), ampicillin (Ap; 100 μg ml^{-1}), rifampicin (Rf; 100 μg ml^{-1}), IPTG (235 μg ml^{-1}) and Xgal (40 μg ml^{-1}) were added to the media.

To grow *V. fischeri* under conditions of a reduced concentration of free iron, we used IM medium, which is identical to the artificial sea water-based basal glycerol medium described by Boettcher and Ruby (1990), except that (i) it was supplemented with 0.3% (w/v) casamino acids; (ii) the Tris buffer was replaced with 87 mM PIPES, pH 6.8; and (iii) $FeCl_3$ was omitted. To make IM-5 medium, 5 μM $FeCl_3$ was added to IM medium. The siderophore indicator medium chrome azurol S (CAS) agar was prepared as described by Lee and Ruby (1994b). The glassware used in low-iron experiments was acid washed in 6 N HCl and rinsed three times with Nanopure (Millipore) water.

Nutritional characterizations were made using an artificial sea water minimal medium (MM) containing 300 mM NaCl, 50 mM $MgSO_4 \cdot 7H_2O$, 10 mM $CaCl_2 \cdot 2H_2O$, 10 mM KCl, 33 mM K_2HPO_4, 0.01 mM $FeSO_4$ and 50 mM Tris-HCl (pH 7.5). This MM was supplemented with either 40 mM glycerol (MM-gly) or 24 mM ribose (MM-rib), and various individual nitrogen sources were added to a final concentration of 10 mM.

Growth yields and growth rates under iron-limiting conditions

Vibrio fischeri stains were grown overnight in LBS and subcultured into IM-5 containing the specific iron chelator ethylenediamine-di(*o*-hydroxyphenyl-acetic acid (EDDHA). Growth rates were determined on cultures shaken at 28°C in IM-5 containing deferrated EDDHA (Neilands, 1994) at a final concentration of between 0 and 200 μM. The maximum growth yields [measured as optical density (OD) at 600 nm] of these cultures were achieved by 39 h. Control cultures containing both 10 μM EDDHA and 25 μM $FeCl_3$ were used to determine whether any characteristic of EDDHA besides iron binding had an effect on the growth rate of *V. fischeri*.

Generation times on different nitrogen sources

The growth rates of strains ES114 and G11 were compared in the defined minimal media MM-gly and MM-rib containing various compounds as sole nitrogen sources. Twenty amino acids, as well as NH_4Cl, $NaNO_3$, cAMP and AMP, were tested. *V. fischeri* was able to use NH_4Cl, cAMP, AMP and the following amino acids: arginine, asparagine, glutamate, glutamine, isoleucine, serine and threonine. Specific growth rates were determined using cells that had been pregrown in MM-gly containing NH_4Cl, washed in MM (without any added

carbon and nitrogen sources) and subcultured into 200 μl of one of several different media contained in 96-well culture plates. The plates were incubated with shaking at 28°C in a plate-reader spectrophotometer, and the OD at 595 nm was determined every hour for 39 h. As a measure of growth rate, the generation time (g) [where $g = (4\ h)/(\ln N_{4\ h} - \ln N_0)$] was determined for the 4 h period over which the growth rate was maximal. The growth yield was considered as the highest OD measured before growth ceased.

Transposon mutagenesis

Transposon mutagenesis was performed as described previously (Graf and Ruby, 1998) using *E. coli* S17–1 (pUTmTn*5*Cm) as the donor strain and *V. fischeri* ESR1 as the recipient. Individual transposon mutants were transferred from the primary selection plates (LBS containing Rf and Cm) to 96-well culture plates containing CAS agar. After incubating the plates for 24 h at 28°C, the mutants were screened for their iron sequestration phenotype.

Characterization and cloning of the transposon insertion

Chromosomal DNA from the *V. fischeri* iron sequestration mutant SP301 was isolated as described by Ausubel *et al*. (1987) and digested with restriction endonucleases. The resulting fragments were separated by agarose gel electrophoresis and transferred onto a nylon membrane that was then prehybridized for 4 h at 42°C in a solution containing 5 × SSC, 50% formamide, 10 × Denhardt's solution and 2.5 mg of herring sperm ml^{-1}. The template DNA for the probe was a gel-purified (GeneClean, Bio101) *Sfi*I fragment of pUTmTn*5*Cm containing the entire Cm^R gene. A ^{32}P-labelled probe was synthesized with a random primer labelling kit using the Klenow fragment (Bethesda Research Laboratories). The membrane was placed in a hybridization solution consisting of one volume of 50% dextran sulphate and four volumes of fresh prehybridization solution containing the labelled probe, and incubated overnight at 42°C. The membrane was then washed three times with 0.1 × SSC containing 0.1% SDS for 20 min at 65°C, followed by three rinses with 6 × SSC before exposure to X-ray film.

A 5 kb *Bgl*II fragment of SP301 DNA that hybridized with the probe was identified by Southern analysis (data not shown) and isolated from the appropriate agarose gel slice by phenol–chloroform extraction. The DNA in this sample was ligated into the dephosphorylated, *Bam*HI-digested vector pBSII. The resulting construct (pJG15) was transformed into cells of *E. coli* XL1-Blue (Stratagene), and transformants were selected by plating, first onto LB agar containing Ap, Xgal and IPTG, and then onto LB agar containing Cm.

Using the same approach, upstream DNA flanking the transposon insertion was cloned from *V. fischeri* strain G11. Chromosomal DNA and pBSII were both double digested with the restriction endonucleases *Not*I and *Xba*I. Ligation of these preparations, followed by transformation of *E. coli* XL1 Blue and selection for Cm^R colonies, yielded pJG26. The plasmids pJG15 and pJG26 were subcloned using standard molecular techniques (Sambrook *et al*., 1989).

Transduction of the transposon

An rp-1 phage lysate was prepared from the siderophore production mutant SP301 as described previously (Visick and Ruby, 1996). This phage stock was used to transduce the wild-type *V. fischeri* strain ES114. Transductants were tested for Cm^R on LBS-Cm agar plates, and for the ability to sequester iron on CAS agar plates.

Isolation of the wild-type glnD *gene from a* V. fischeri *genomic library*

Using a combination of Southern hybridizations with a 2 kb *Eco*RI fragment of *V. fischeri glnD* carried in pJG28 and DNA sequence analysis, we determined that a 7 kb *Eco*RI chromosomal DNA fragment contains the entire *V. fischeri glnD* gene (data not shown). Chromosomal DNA of ES114 was digested with *Eco*RI, and the resulting fragments were size fractionated and cloned into the *Eco*RI site of pBSII. Approximately 2000 *E. coli* clones carrying this library were screened by colony hybridization with the 2 kb *Eco*RI fragment of pJG28, and two positive colonies were obtained. The insert of one of these clones was subcloned as an *Eco*RI–*Bgl*II fragment into the corresponding sites of the *V. fischeri* conjugative vector pVO8 (Visick and Ruby, 1997), creating pJG40.

Nucleotide sequence accession number

The nucleotide sequence of the *V. fischeri glnD* gene has been submitted to the DDBJ/EMBL/GenBank databases under accession number AF152563.

Colonization assays

The *E. scolopes* light organ colonization assay used has been described previously (Graf *et al*., 1994). Newly hatched juvenile squids were exposed for 12 h to approximately 10^3 *V. fischeri* cells ml^{-1} sea water in glass vials. After this initial exposure, the sea water was replaced with *V. fischeri*-free sea water every 24 h. At daily intervals, the number and type of bacteria present within the light organs of juvenile squids were determined by spreading dilutions of homogenized animals on SWT agar (Ruby and Asato, 1993). After an overnight incubation at 23°C, colonies had arisen and were counted and examined for morphological uniformity. Five isolates from each animal were tested for the expected antibiotic resistance, as well as for the ability to form haloes on CAS agar plates. The stability of the transposon during the course of these experiments was confirmed by Southern hybridization of DNA isolated from five isolates obtained from homogenates of light organs that had been colonized for 72 h.

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