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Bright luminescence of *Vibrio fischeri* aconitase mutants reveals a connection between citrate and the Gac/Csr regulatory system

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Summary

The Gac/Csr regulatory system is conserved throughout the γ-proteobacteria and controls key pathways in central carbon metabolism, quorum sensing, biofilm formation and virulence in important plant and animal pathogens. Here we show that elevated intracellular citrate levels in a *Vibrio fischeri* aconitase mutant correlate with activation of the Gac/Csr cascade and induction of bright luminescence. Spontaneous or directed mutations in the gene that encodes citrate synthase reversed the bright luminescence of aconitase mutants, eliminated their citrate accumulation and reversed their elevated expression of CsrB. Our data elucidate a correlative link between central metabolic and regulatory pathways, and they suggest that the Gac system senses a blockage at the aconitase step of the tricarboxylic acid cycle, either through elevated citrate levels or a secondary metabolic effect of citrate accumulation, and responds by modulating carbon flow and various functions associated with host colonization, including bioluminescence.

Introduction

Vibrio fischeri is a bioluminescent γ-proteobacterium that enters a light-organ symbiosis with the Hawaiian bobtail squid, *Euprymna scolopes* (Stabb, 2006). Bacterial bioluminescence is a key element of this symbiosis, as light is used by the nocturnal host (Jones and Nishiguchi, 2004; Stabb and Millikan, 2009), bioluminescence is a colonization factor for *V. fischeri* (Visick *et al*., 2000; Bose *et al*., 2008; Koch *et al*., 2014) and light emission elicits responses in host tissue (Visick *et al*., 2000; Chun *et al*., 2008; Tong *et al*., 2009). *V. fischeri* induces bioluminescence most highly during symbiotic infection, prompting interest in its regulation.

Bioluminescence is controlled in part by the LuxR-LuxI pheromone-mediated regulatory system, which is often referenced as an archetype of ‘quorum sensing’, although other regulation is critical as well. LuxI synthesizes the pheromone *N*-(3-oxohexanoyl)-L-homoserine lactone (3OC6) (Eberhard *et al*., 1981), which upon reaching a threshold concentration binds LuxR to activate transcription of *luxICDABEG* and induce luminescence (Nealson *et al*., 1970; Devine *et al*., 1989). Although 3OC6 levels correlate with cell density, *V. fischeri* cells growing in culture are ~ 1000-fold less luminescent and accumulate less 3OC6 than cells in the host light organ even at similar high cell density (Boettcher and Ruby, 1995), illustrating that other regulators exert important control over the *lux* system as well.

To investigate *lux* regulation, Lyell *et al*. (2010) screened mutants of *V. fischeri* ES114 for bright luminescence in culture. They identified mutants > 100-fold brighter than wild type with transposon insertions in *acnB* (VF_2158), which is the sole gene in *V. fischeri* that encodes aconitase, the tricarboxylic acid (TCA) cycle enzyme responsible for converting citrate to isocitrate (Fig. S1A). Although bright mutants were isolated with insertions in 13 other loci, none were affected in other TCA cycle enzymes (Lyell *et al*., 2010).

In some systems, a post-transcriptional regulatory role for aconitase has been suggested, whereby enzymatically inactive aconitase apoproteins lacking iron-sulfur clusters bind specific mRNAs and affect either the turnover or translation of target transcripts (Tang and Guest, 1999; Tang *et al*., 2002; 2004; 2005; Austin and Maier, 2013). The active aconitase enzyme contains an iron-sulfur cluster, but apo-aconitase is implicated in post-transcriptional regulation upon loss of the iron-sulfur cluster due to oxidative stress or low iron (Tang and Guest, 1999; Varghese *et al*., 2003; Thorgersen and Downs, 2009), conditions that are often found in host tissue, including the *E. scolopes*

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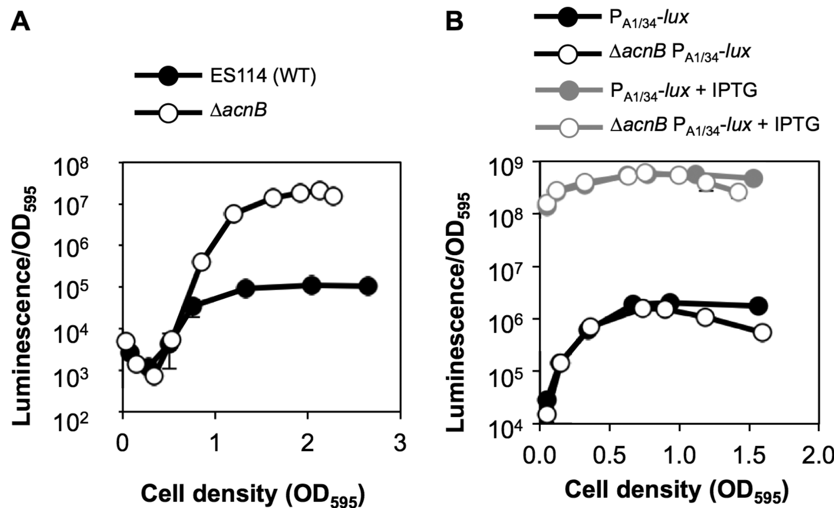

Fig. 1. Effect of a mutant Δ*acnB* allele on luminescence requires native *lux* regulation. Specific luminescence is shown for aerobic cultures in SWTO medium.
A. Comparison of wild-type (WT) ES114 (filled circles) and Δ*acnB* mutant JB26 (open circles). Error bars indicate standard deviation (though some cannot be seen) ($n = 3$).
B. Comparison of JB22 (*lacI*[q] $P_{A1/34}$-*luxCDABEG*) (filled symbols) and its isogenic Δ*acnB* mutant, ANS29 (open symbols) without (black lines) or with (gray lines) 0.2 mM IPTG. Error bars indicate standard deviation (though some cannot be seen) ($n = 2$).

light organ (Visick and Ruby, 1998; Small and McFall-Ngai, 1999; Graf and Ruby, 2000; Septer *et al.*, 2011). The results of Lyell *et al.* (2010) might reflect apo-aconitase-regulating luminescence post-transcriptionally, but there are other possibilities. For example, blocking the TCA cycle might cause a metabolic shift that either affects the availability of substrates for luciferase or triggers a regulatory response leading to a change in expression of the *lux* operon.

A candidate for connecting *acnB* mutants with *lux* regulation is the GacS/GacA two-component system, which is conserved among many γ-proteobacteria and often plays a critical role in host colonization (Lapouge *et al.*, 2008). In *V. fischeri*, GacA regulates many colonization factors, including bioluminescence (Whistler and Ruby, 2003; Whistler *et al.*, 2007). Studies in *Pseudomonas* suggest that activation of the sensor kinase GacS may be linked to an imbalance in TCA cycle intermediates (Takeuchi *et al.*, 2009), and the periplasmic-loop region of GacS is not required for signal reception (Zuber *et al.*, 2003), consistent with GacS activity being linked to a cytoplasmic metabolite. These reports led us to speculate that perturbing the TCA cycle might regulate luminescence by modulating the Gac system.

Current understanding of how GacS/GacA (BarA/UvrY) and their partners CsrA/CsrB (RsmA/RsmB) regulate gene expression (Lapouge *et al.*, 2008) is based largely on studies in *Pseudomonas* species (Rich *et al.*, 1994; Brencic *et al.*, 2009) and *Escherichia coli* (Romeo *et al.*, 1993; Sabnis *et al.*, 1995; Romeo, 1998; Wei *et al.*, 2000; Pernestig *et al.*, 2001), although this system has been examined in the *Vibrionaceae* and *V. fischeri* (Lenz *et al.*, 2005; Kulkarni *et al.*, 2006; Ballok, 2007; Williams *et al.*, 2012). It is thought that in response to an activating signal, the membrane-bound sensor kinase GacS phosphorylates the DNA-binding response regulator GacA. GacA-phosphate then activates transcription of two CsrB regulatory RNAs, which contain multiple-binding sites for the post-transcriptional carbon storage regulator, CsrA. The CsrB RNAs thereby titrate CsrA away from target mRNAs that it would otherwise control (Fig. S2). Regardless of the mechanism by which Gac regulates luminescence in *V. fischeri*, we considered the possibility that the effect of an *acnB* mutation on luminescence might be due to a metabolic signal perceived by GacS leading to increased CsrB expression.

The goal of this study was to explore the unexpected bright luminescence of *V. fischeri* aconitase mutants. By examining whether the effect of aconitase on luminescence required (i) apo-aconitase, (ii) carbon flow into the TCA cycle upstream of aconitase, (iii) the native *lux* promoter or (iv) specific regulators such as GacS, we were able to systematically test alternative explanations for this phenotype.

Results

Bright luminescence of the ΔacnB mutant requires native luxICDABEG regulation

To examine why aconitase mutants are brighter than wild type, we first tested whether the loss of aconitase causes enhanced bioluminescence through a metabolic effect that influences luciferase's activity. Luciferase consumes oxygen and reducing equivalents, and we considered the possibility that loss of aconitase might enhance luminescence by altering the availability of these substrates. An in-frame deletion of the *acnB* gene (VF_2158) generated a mutant that was ~ 200 times brighter than the wild-type parent (Fig. 1A), which is similar to the bright luminescence previously described for *acnB*::Tn mutants (Lyell *et al.*, 2010). However, in a strain that has a non-native isopropyl β-D-thiogalactopyranoside (IPTG)-inducible promoter

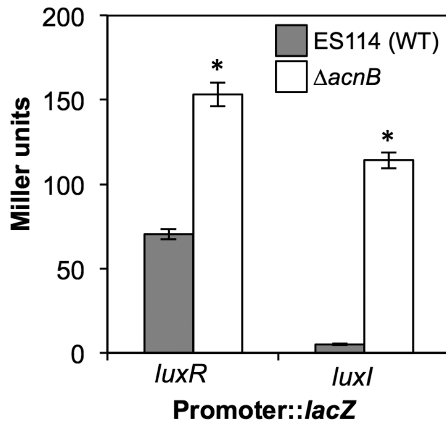

Fig. 2. Deleting *acnB* affects expression of *luxI* and *luxR* transcriptional reporters. LacZ activity was measured in strains harboring plasmid-based P_{luxR}- and P_{luxI}-*lacZ* transcriptional reporters on pJLB170 and pJLB171, respectively. Cultures of wild-type (WT) ES114 (gray) or the Δ*acnB* mutant JB26 (white) harboring pJLB170 or pJLB171 were grown in aerobic shake flasks at 24°C in SWTO medium. ß-galactosidase assays were performed on cells harvested at an OD_{595} of 2.0. Error bars indicate standard deviation ($n = 3$). Asterisks indicate statistical significance with a P-value < 0.006 using a Student's *t*-test comparing activity in the Δ*acnB* mutant with that of the WT for each reporter. These data are representative of at least three independent experiments.

driving the *luxCDABEG* genes in their native position on chromosome II, introducing the same Δ*acnB* allele did not increase luminescence relative to the parent either in the presence or absence of IPTG (Fig. 1B). Moreover, plasmid-based *lacZ* transcriptional reporters indicated increased *luxR* and *luxI* promoter activity in the Δ*acnB* mutant compared with wild type (Fig. 2). Although the Δ*acnB* allele has a smaller effect on the P_{luxI} reporter than on luminescence itself, this is a typical relationship between luminescence and *lux* reporters or mRNA (Antunes *et al*., 2007; Bose *et al*., 2007; Lyell *et al*., 2010; Perez *et al*., 2011; Septer and Stabb, 2012), which is most thoroughly discussed by Perez *et al*. (2011). Taken together, these data indicate the bright luminescence of the Δ*acnB* mutant reflects a regulatory effect involving native *lux* regulation and cannot be accounted for simply by a physiological effect influencing substrate availability for luciferase.

Bright luminescence of the ΔacnB mutants appears to involve TCA cycle function during respiration

The Δ*acnB* mutant was brighter than wild type under aerobic conditions, and similar luminescence differences were observed when the strains were grown anaerobically supplemented with trimethylamine *N*-oxide (TMAO) as an alternative electron acceptor (Fig. 3). However, when grown fermentatively, there was no significant difference in luminescence of the Δ*acnB* mutant relative to wild type (Fig. 3), even when the medium was supplemented with 50 ng ml^{-1} 3OC6 pheromone to stimulate *lux* expression (data not shown). Thus, the effect of aconitase on luminescence was most apparent under conditions where, presumably, carbon would be directed toward the TCA cycle, thereby producing reducing equivalents to support respiration. Although much of *V. fischeri*'s physiology remains to be elucidated, bioinformatic analysis of the ES114 genome indicated that it encodes all the enzymes necessary for a complete TCA cycle and glyoxylate shunt (Fig. S1A), as well as respiratory oxidases (Dunn *et al*., 2010).

Suppressor mutations in gltA *restore dim luminescence to ΔacnB mutants*

When grown aerobically in a rich medium, the Δ*acnB* mutant was brighter than wild type but also displayed slower growth rates (Fig. 4A) and lower growth yield (data not shown) than did the wild type. It may seem surprising that a mutant lacking aconitase could grow at all; however, even under conditions that support respiration, wild-type *V. fischeri* apparently generates energy from fermentative pathways and releases fermentation acids. When deprived of any fermentable substrates, growth of the *acnB* mutant was blocked or more severely attenuated. Moreover, our aconitase mutants had a strict requirement for glutamate in the medium, consistent with AcnB's role in the TCA cycle (Fig. S1A) and the bioinformatic prediction that glutamate synthesis would require alpha-ketoglutarate as a precursor. Glutamate in the medium might also promote growth of the *acnB* mutant by enabling it to use the combined activi-

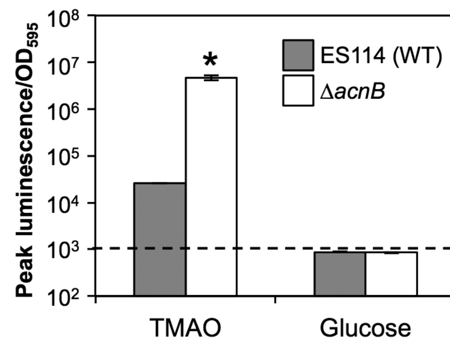

Fig. 3. Effect of Δ*acnB* mutation on anaerobically grown cells supplemented with an electron acceptor or fermentable carbon source. Peak specific luminescence for wild-type (WT) ES114 (gray) and Δ*acnB* mutant JB26 (white) cultures grown anaerobically in SWTO medium supplemented with 40 mM TMAO or 20 mM glucose. Dashed line indicates limit of detection (background) for luminescence measurements. Error bars indicate standard deviation (though some cannot be seen) ($n = 2$), and the asterisk indicates statistical significance with a P-value < 0.0001 using a Student's *t*-test comparing the Δ*acnB* mutant with the WT within each treatment. These data are representative of at least three independent experiments.

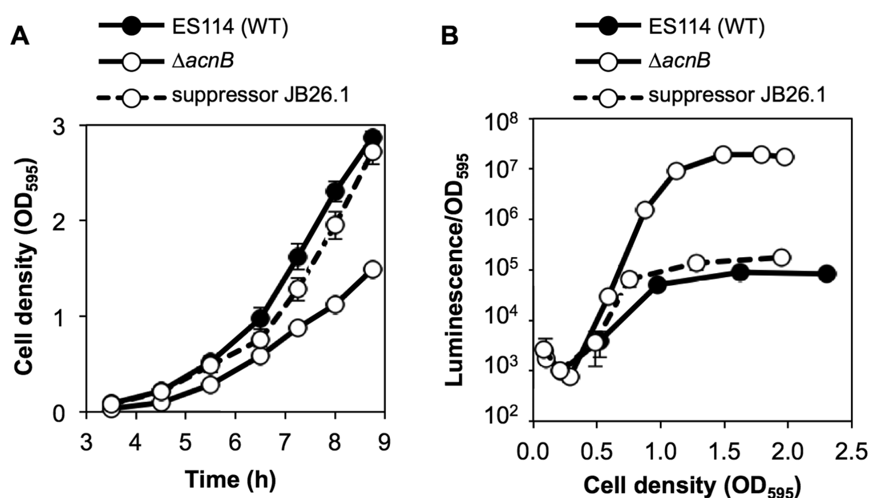

Fig. 4. Growth and luminescence phenotypes of the Δ*acnB* suppressor mutant JB26.1. Cell density (OD_{595}) over time (A) and luminescence as a function of cell density (B) for cultures of wild-type (WT) ES114 (filled circles), Δ*acnB* mutant JB26 (open circles) and Δ*acnB* suppressor mutant JB26.1 (open circles, dashed line) grown aerobically in SWTO. In each panel, data are representative of at least three independent experiments, and error bars indicate standard deviation for triplicate biological replicates (though some cannot be seen).

ties of aspartate aminotransferase (VF_A0283) and aspartate ammonia lyase (VF_2353) to operate an abridged TCA cycle lacking citrate (Fig. S1B).

As previously noted for *acnB*::Tn mutants (Lyell *et al.*, 2010), spontaneous mutants suppressing the Δ*acnB* slow-growth phenotype arose quickly during aerobic growth on rich media. We estimated these suppressors arose at a frequency of ~ 10^{-4}, resulting in wild type-like rapid growth and dim luminescence (Fig. 4A and B). Because LeuCD is in the aconitase superfamily, we initially focused our efforts to define the sites of suppression within suppressors isolated in the Δ*leuCD* Δ*acnB* background of strain ANS28, to eliminate the possibility of mutations in *leuCD*-restoring aconitase activity. However, we later found no evidence that *leuCD* affects suppression frequency or the site of suppression. Moreover, despite their rapid growth on rich media, all of the suppressors retained the glutamate auxotrophy of the Δ*acnB* parent, suggesting they remain blocked at this step in the TCA cycle.

To identify the site of suppression in the Δ*acnB* suppressor mutants, we first employed whole-genome resequencing of suppressors isolated in a Δ*leuCD* Δ*acnB* background. In each of the six independent suppressor mutants, we identified mutations in the *gltA* gene (VF_0818), which encodes citrate synthase (Table S1). Sequencing the *gltA* locus in strain JB26.1, a spontaneous suppressor isolated from the homogenate of a squid inoculated with JB26 (*leuCD*$^{+}$ Δ*acnB*) similarly revealed a mutation in *gltA* (Table S1), suggesting that there is selective pressure favoring the growth of such suppressors during symbiotic colonization as well. We inoculated fresh aposymbiotic *E. scolopes* hatchlings and confirmed that suppressor JB26.1 colonizes the host better than its parent, achieving wild-type colonization levels in the host (Fig. S3).

Based on the mutations recovered in *gltA* (Table S1), we hypothesized that loss of citrate synthase suppressed the phenotypes associated with the Δ*acnB* mutation. To test this possibility, we deleted *gltA* and introduced the Δ*acnB* mutation to generate a Δ*gltA* Δ*acnB* double mutant. Consistent with our model and our earlier data, the Δ*acnB* mutation had no influence on growth or luminescence in the Δ*gltA* background (Fig. 5). The Δ*gltA* mutation alone did not have a statistically significant effect on luminescence relative to wild type (Fig. 5). However, bright luminescence was observed in the Δ*gltA* Δ*acnB* mutant when *gltA* was complemented *in trans* (Fig. 5), confirming that a functional citrate synthase is required for bright luminescence of the Δ*acnB* mutant. Thus, in contrast to instances where apo-aconitase itself functions directly as a regulator, our data suggest the regulatory effect of the Δ*acnB* allele on luminescence in *V. fischeri* results from blocking the TCA cycle immediately after citrate synthase.

Citrate accumulation in ΔacnB mutants correlates with luminescence

The previous study by Lyell *et al.* (2010) did not identify bright mutants with transposon insertions blocking the TCA cycle at any enzymes other than aconitase, and as shown above, the effect of the Δ*acnB* allele was dependent on citrate synthase. We therefore hypothesized that citrate accumulates in the Δ*acnB* mutant during respira-

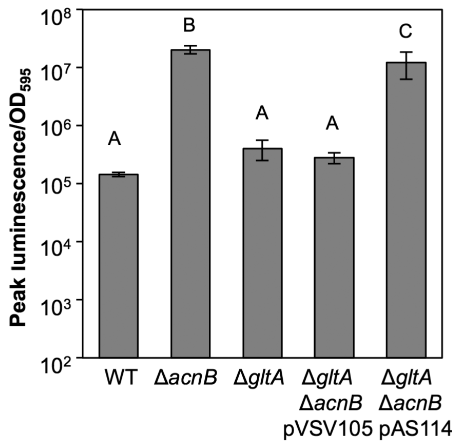

Fig. 5. The bright luminescence of Δ*acnB* mutants is dependent on *gltA*. Peak specific luminescence for *Vibrio fischeri* cultures grown aerobically in SWTO. Strains tested include wild-type (WT) ES114, Δ*acnB* mutant JB26, Δ*gltA* mutant ANS60 and Δ*acnB* Δ*gltA* mutant ANS61 carrying either the empty vector pVSV105 or complemented with the *gltA*-containing vector pAS114. Error bars indicate standard deviation ($n = 3$). Uppercase letters shared between bars indicate no statistically significant difference ($P > 0.9$), whereas different letters indicate significant difference ($P < 0.01$), based on a one-way analysis of variance (ANOVA) with Fisher's least significant difference comparison. These data are representative of at least three independent experiments.

tion and that these elevated citrate levels may directly or indirectly influence luminescence through an unknown pathway. Levels of intracellular citrate were elevated in aerobic cultures of the Δ*acnB* mutant, whereas levels in the wild type, Δ*gltA* mutant and a Δ*acnB gltA* mutant were all below the limit of detection for the assay (Table 1). Moreover, when cultures were grown anaerobically with TMAO to support respiration, the Δ*acnB* mutant also accumulated high concentrations of citrate, but citrate accumulation was barely above the limit of detection in the Δ*acnB* mutant when cultures were grown fermentatively (Table 1). Thus, the accumulation of citrate correlates with conditions under which bright luminescence was observed for Δ*acnB* mutants. Specifically, luminescence (Figs 3 and 5) and citrate (Table 1) were increased in Δ*acnB* mutants with a functional citrate synthase gene under conditions supporting respiration.

Citrate-mediated iron limitation does not account for bright luminescence in ΔacnB mutants

Iron limitation can lead to increased luminescence of *V. fischeri* (Haygood and Nealson, 1985), and adding exogenous iron chelators, notably including citrate, can lead to brighter luminescence (Septer *et al.*, 2013). However, our data suggest this iron chelation effect is not responsible for the enhanced luminescence of a Δ*acnB* mutant. We previously found that adding citrate to cultures increased luminescence less than 10-fold, that this effect was dependent on Fur-mediated regulation of *litR* and that it was reversible by adding iron (Septer *et al.*, 2013). By contrast, deleting *acnB* increases luminescence 100- to 1000-fold, and this effect is neither reversible by adding iron (Fig. 6A) nor dependent on *litR* (Fig. 6B). We considered the possibility that the Δ*acnB* mutant is simply more severely limited for iron than cells treated with exogenous citrate, and that adding iron to the medium (Fig. 6A) was insufficient to overcome the iron-limitation effect. To test this alternative possibility, we took advantage of an established iron-responsive, Fur-regulated transcriptional reporter on plasmid pAKD912 (Septer *et al.*, 2011; 2013). We found that addition of iron to the Δ*acnB* mutant reversed the iron-limitation response of this Fur-controlled reporter (Fig. S4), under the same conditions where bright luminescence was not reversed (Fig. 6A). Thus, our data suggest that deletion of *acnB* affects luminescence primarily through a mechanism independent of iron limitation.

GacS is required for bright luminescence in the ΔacnB mutant

We next considered whether the Gac/Csr system might connect the effects of the Δ*acnB* mutation with luminescence regulation, because GacA regulates luminescence (Whistler and Ruby, 2003) and previous studies were consistent with a TCA-cycle intermediate activating GacS (Zuber *et al.*, 2003; Takeuchi *et al.*, 2009). We hypothesized that elevated citrate levels, or a related secondary

Table 1. Citrate accumulation in *Vibrio fischeri* strains.

Genotype	Growth conditions[a]	Nanomole citrate per 10^9 CFU[b]
WT	Aerobic	bd
Δ*gltA*	Aerobic	bd
Δ*acnB*	Aerobic	381
Δ*acnB gltA*	Aerobic	bd
Δ*acnB* Δ*gacS*	Aerobic	bd
Δ*acnB* Δ*csrB1* Δ*csrB2*	Aerobic	23.6
WT	Fermentative	bd
Δ*gltA*	Fermentative	bd
Δ*acnB*	Fermentative	0.5[c]
Δ*acnB gltA*	Fermentative	bd
WT	Anaerobic respiration	bd
Δ*gltA*	Anaerobic respiration	bd
Δ*acnB*	Anaerobic respiration	852
Δ*acnB gltA*	Anaerobic respiration	bd

a. *V. fischeri* was grown in SWTO medium either in aerobic shake flasks or in anaerobic bottles supplemented with 20 mM glucose (fermentative) or 40 mM TMAO (anaerobic respiration). Data are representative of three independent experiments.
b. 'bd' indicates samples had citrate levels below detection for this assay (< 0.5 nmol).
c. Citrate was detectable in one of the three independent experiments for the Δ*acnB* mutant grown under fermentative conditions, suggesting citrate levels are around the limit of detection for this assay.

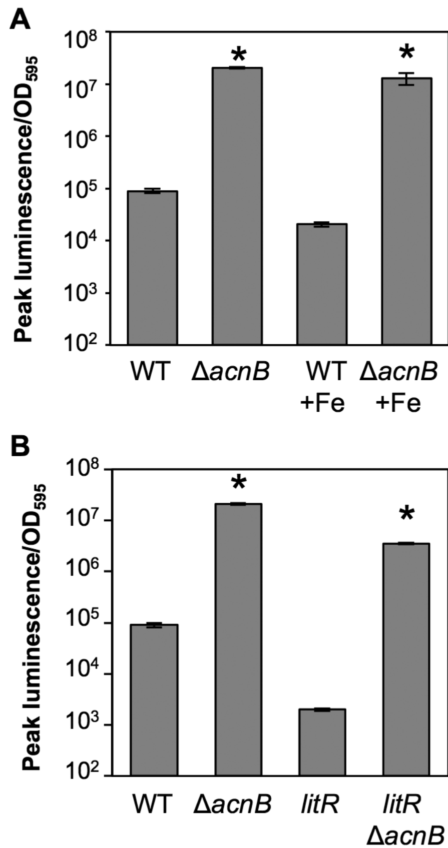

Fig. 6. Effects of citrate-mediated iron limitation on the Δ*acnB* mutant. Peak specific luminescence of aerobic cultures grown in SWTO.
A. Comparison of wild-type (WT) ES114 and the Δ*acnB* mutant JB26 with or without 200 μM $FeSO_4$.
B. Effect of Δ*acnB* allele compared in WT and *litR* mutant backgrounds. Strains include ES114 (WT), JB26 (Δ*acnB*), JB19 (*litR*::*erm*) and ANS79 (Δ*acnB litR*::*erm*). In each panel, error bars indicate standard deviation (some too small to visualize) ($n = 3$). Asterisks indicate $P < 0.01$ with a Student's *t*-test comparing the Δ*acnB* with the parent strain. Each panel is representative of at least three independent experiments.

metabolic consequence, in a Δ*acnB* mutant are sensed by GacS resulting in modulation of GacA, CsrBs and CsrA, ultimately leading to direct and/or indirect induction of luminescence. To begin testing this hypothesis, we deleted the *gacS* sensor kinase gene (VF_2082) and introduced the Δ*acnB* mutation into this background. In the absence of *gacS*, the Δ*acnB* mutation did not result in the same bright luminescence observed in the wild-type background (Fig. 7). Thus, *gacS* is required for bright luminescence in the Δ*acnB* mutant.

The ΔacnB mutant has elevated levels of CsrB RNAs

Putative GacA-binding sites were identified upstream of both *csrB* genes in *V. fischeri* (Kulkarni *et al.*, 2006), and Ballok reported that transcriptional reporters specific to *csrB1* or *csrB2* have lower expression in a *gacA* mutant (Ballok, 2007). These previous findings suggest that, consistent with observations in other organisms, GacA activates expression of CsrBs in *V. fischeri*. Based on our hypothesis above, we predicted that strains with endogenous citrate accumulation would increase expression of the *csrB* regulatory RNAs. We tested this idea using CsrB-specific quantitative real-time polymerase chain reaction (qRT-PCR) and RNA isolated from wild-type, Δ*acnB* and Δ*acnB* suppressor mutant cultures. Primers specific for *csrB1* or *csrB2* cDNA had between one and three mismatches to the non-target *csrB*. Given their similar sequences and product sizes, we cannot rule out the possibility that our primer pair matching *csrB1* also amplified *csrB2* cDNA and vice versa; however, that outcome would not meaningfully alter our conclusions. Consistent with our model, the Δ*acnB* mutant had three- to fourfold higher levels of CsrB RNA, using either primer pair, compared with the wild type (Fig. 8A). Moreover, the Δ*acnB gltA* suppressor mutant had CsrB levels lower than those in the wild type (Fig. 8A). Considering some intracellular citrate should be present in wild type, whereas the *gltA* mutant presumably has virtually none, CsrB levels correlate with expected relative citrate levels (Δ*acnB* > WT > Δ*acnB gltA*).

CsrB regulatory RNAs are required for bright luminescence of the ΔacnB mutant

Having shown elevated CsrB levels in the Δ*acnB* mutant, we predicted that bright luminescence of the Δ*acnB*

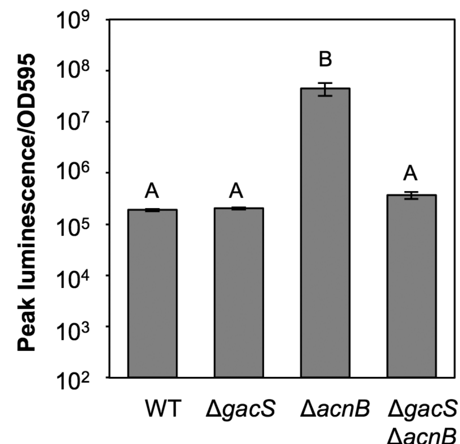

Fig. 7. The role of *gacS* in bright luminescence of the Δ*acnB* mutant. Peak specific luminescence for *Vibrio fischeri* cultures of ES114 [wild type (WT)], ANS64 (Δ*gacS*), JB26 (Δ*acnB*) and ANS74 (Δ*acnB* Δ*gacS*) grown aerobically in SWTO. Uppercase letters shared between bars indicate no statistically significant difference ($P > 0.9$), whereas different letters indicate significant difference ($P < 0.0001$), based on a one-way analysis of variance (ANOVA) with Fisher's least significant difference comparison. Data are representative of at least three independent experiments. Error bars indicate standard deviation ($n = 3$).

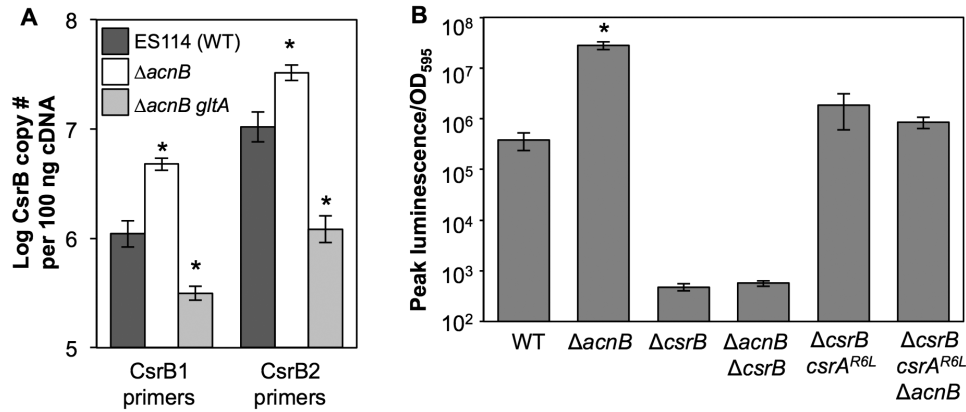

Fig. 8. The role of CsrBs in bright luminescence of the Δ*acnB* mutant.
A. CsrB copy numbers for RNA isolated from cultures of wild type (WT) (dark gray), Δ*acnB* (white) and aconitase mutant suppressor, Δ*acnB gltA* (light gray), grown aerobically in SWTO medium at 24°C with shaking. Cells were harvested at an OD_{595} of ~ 1.0 for RNA isolation. Primer sets were exact matches to CsrB1 or CsrB2, with between one and three mismatches to the other CsrB. We cannot rule out cross-hybridization and amplification of the non-target CsrB reflected in the Y-axis values. Values for each treatment are the average of biological triplicates. Error bars indicate standard error and asterisks denote statistically significant difference from WT; $P \leq 0.03$ using a Student's *t*-test on log-transformed data.
B. Peak specific luminescence of cultures grown aerobically in SWTO medium. Δ*csrB* indicates deletion of both *csrB1* and *csrB2*. Strains tested include ES114 (WT), JB26 (Δ*acnB*), ANS62 (Δ*csrB1* Δ*csrB2*), ANS70 (Δ*acnB*, Δ*csrB1* Δ*csrB2*), ANS62.1 (Δ*csrB1* Δ*csrB2* *csrA*^{R6L}) and ANS81 (Δ*csrB1* Δ*csrB2* *csrA*^{R6L} Δ*acnB*). Error bars indicate standard deviation (though some are too small to visualize) ($n = 3$), and the asterisk denotes statistically significant difference from the respective *acnB*$^{+}$ parent strain; $P < 0.001$ using a Student's *t*-test. Data in B are representative of at least three independent experiments.

mutant would be dependent on these CsrBs. To test this prediction, we constructed a *csrB* null mutant (Δ*csrB1* Δ*csrB2*) and moved the Δ*acnB* mutation into this background. As predicted, the Δ*acnB* mutation did not have any effect on luminescence in the *csrB* null background (Fig. 8B).

We considered the possibility that the *csrB* genes might be required to support bright luminescence for physiological rather than regulatory reasons, for example, by affecting the availability of substrates for luciferase. It seemed especially prudent to consider this possibility, because the *csrB* null mutant had obvious growth defects similar to those of the *acnB* mutant shown in Fig. 4, but with cultures in rich media achieving a slightly lower final OD_{595} of ~ 1.4. We therefore generated a *csrB* null mutant in a strain that has *luxCDABEG* under control of an IPTG-inducible promoter. Despite a growth defect characteristic of a *csrB* null mutant, this strain produced bright luminescence like that of the parent strain (Fig. S5). Similarly, deleting *arcA* enhances luminescence (Bose *et al*., 2007), and this effect was still evident in a *csrB* null background (data not shown). Thus, *csrB* null mutants are metabolically capable of bright luminescence, and our data suggest the *csrB* dependence for bright luminescence in *acnB* mutants is a specific effect of native *luxICDABEG* regulation.

Although *csrB* null mutants were capable of bright luminescence, we wondered whether their attenuated growth might specifically interfere with the effect on luminescence mediated by the Δ*acnB* allele. To test this possibility, we grew the *csrB* null mutant under respiratory conditions and isolated faster growing suppressor mutants, which we suspected might have partial-loss-of-function mutations in *csrA*, as do *gacA* suppressor mutants (Ballok, 2007). Sequencing of the *csrA* gene in *csrB* null suppressor mutant ANS62.1 revealed a single base change resulting in an arginine to leucine substitution at the sixth residue in CsrA. Unlike its *csrB* null parent, strain ANS62.1 (*csrB* null *csrA*^{R6L}) showed wild type-like growth (data not shown), yet the Δ*acnB* mutation had no effect on luminescence in this background (Fig. 8B). Taken together, our data indicate that an intact Csr system is required for the effect of the Δ*acnB* mutation on luminescence, regardless of attenuated or wild type-like growth rates.

Role of Csr system in regulating luminescence

In addition to illustrating that the effect of the Δ*acnB* allele on luminescence is dependent on the *csrB* genes, the values of the luminescence data in Fig. 8B also provide insight into the Gac/Csr system in *V. fischeri* and the earlier observation that *gacA* mutants are dim (Whistler and Ruby, 2003). If GacA functions primarily by promoting transcription of the *csrB* genes, then we would predict that the *csrB* null mutant would likewise be dimmer than wild type. Consistent with this prediction, the *csrB* null mutant is 800-fold dimmer than ES114 (Fig. 8B). Similarly, if the CsrBs function primarily by titrating CsrA to relieve direct or indirect repression of *lux*, then a partial-loss-of-function

csrA mutant would be brighter than the parent. Indeed, the *csrA*^{R6L} allele increased luminescence ~ 4000-fold in the *csrB* null background (Fig. 8B). Although the mechanism(s) connecting Csr to luminescence require further investigation, the data in Fig. 8B are consistent with CsrA repressing luminescence either directly (Ballok, 2007) and/or indirectly (Williams *et al*., 2012).

GacS but not CsrB RNAs are required for citrate accumulation in ΔacnB mutants

We hypothesized that Gac and Csr mediate a response to citrate accumulation; however, the epistatic effects of *csrB* and *gacS* mutations on the aconitase mutant would also be consistent with Gac and Csr alternatively or additionally mediating citrate accumulation itself. We did not detect citrate accumulation in Δ*acnB* mutants also lacking *gacS*, suggesting that Gac regulates citrate synthesis and that *gacS* is required for citrate accumulation in the Δ*acnB* mutant (Table 1). Importantly though, the Δ*acnB* mutant lacking *csrB* genes did accumulate elevated levels of citrate (Table 1), yet this strain did not have elevated luminescence (Fig. 8B).

Discussion

Upon investigating the mechanism underlying the bright luminescence of *V. fischeri* aconitase mutants, we identified an intriguing link between aconitase, citrate (the substrate for aconitase) and regulation mediated by the Gac/Csr system. First, we found that genetic and physiological conditions correlating with accumulation of citrate also corresponded with bright luminescence. Although citrate and other chelators can influence luminescence through a *fur*- and *litR*-dependent mechanism (Septer *et al*., 2013), our results here indicate that such a mechanism cannot explain most of the enhancement of luminescence observed in an aconitase mutant. However, we did observe that the effect of an *acnB* mutation on luminescence was entirely dependent on *gacS* and *csrB*s.

Our study indicated that the Gac/Csr regulatory cascade in *V. fischeri* appears to follow a predicted model (Fig. S2) based on what has been described in other organisms, but with an unexpected finding that is important for interpretation of our results: *gacS* and *gacA* mutants have dissimilar phenotypes. Compared with wild type, *gacA* mutants grow more slowly, are less luminescent and do not colonize the host as well (Whistler and Ruby, 2003); however, the Δ*gacS* mutant had wild type-like growth, luminescence (Fig. 7) and symbiotic colonization (Fig. S3). These data indicate that there is a GacS-independent element to the Gac/Csr system and suggest that either unphosphorylated GacA can activate *csrB* expression, or that GacA may be phosphorylated by acetyl-phosphate as described for *E. coli* (Tomenius *et al*., 2005) or by another sensor kinase in the absence of GacS. Suzuki *et al*. similarly reported that in *E. coli*, mutating *uvrY* (the *gacA* homolog) had more severe effects on *csrB* transcription than did mutating *barA* (the *gacS* homolog) (Suzuki *et al*., 2002).

Other results were consistent with the prevailing model of Gac/Csr function (Fig. S2) and clearly connected the luminescence phenotype of the *acnB* mutant with the Gac/Csr system. We found that the *csrB* null mutant did resemble the reported phenotypes of a *gacA* mutant, and these phenotypes, including dim luminescence, could be suppressed by a point mutation in *csrA*. The more novel and unexpected findings here were that the effect of the Δ*acnB* mutation on luminescence correlated with an increase in CsrB levels (Fig. 8A) and was eliminated in the *csrB* null background (Fig. 8B). Even when a point mutation in *csrA* suppressed the overall dimness of the *csrB* null strain, luminescence in the resulting mutant was unaffected by deleting *acnB* (Fig. 8B). Taken together, our results are consistent with a model whereby the Gac/Csr system mediates the effect of *acnB* on *lux* regulation.

The effects of the Δ*acnB* mutation were also strongly correlated with citrate accumulation, and were abrogated by mutations in *gltA* that prevented citrate accumulation. Further research is necessary to determine the mechanism(s) reflected in this observation and its role, if any, in symbiotic colonization. It is possible that citrate itself stimulates the Gac system, either by influencing GacS or through the apparent GacS-independent function of GacA and CsrB noted above. On the other hand, a metabolomic comparison of Δ*acnB* and Δ*acnB* Δ*gltA* mutants might reveal differences other than citrate accumulation, which in turn could provide the direct link to the Gac/Csr system. For example, citrate can inhibit pyruvate kinase, so elevated citrate might influence GacS activation indirectly by affecting phosphoenolpyruvate or pyruvate levels. Alternatively, buildup of oxaloacetate, which is a substrate for GltA, rather than citrate, might alter carbon flow in the absence of a canonical TCA cycle (Fig. S1B). The Gac/Csr system could respond to such an effect rather than to the accumulation, or not, of citrate per se.

Although much is known about the genes regulated by the Gac/Csr cascade in many organisms (Lapouge *et al*., 2008; Timmermans and Van Melderen, 2010), the signal(s) responsible for modulating this system have remained unclear. Dubuis and Haas showed that a signal produced by *Vibrio harveyi* activates Gac in *Pseudomonas fluorescens* (Dubuis and Haas, 2007), indicating the signal may be conserved. Consistent with our results, others have proposed that GacS activation may be linked to an imbalance in TCA cycle intermediates (Takeuchi *et al*., 2009), while another study suggested the signal could be cytoplasmic (Zuber *et al*., 2003). Findings

in *Pseudomonas* showed that synthesis of the Gac-activating signal required the Gac two-component system itself (Kay *et al*., 2005), consistent with our observation that citrate did not accumulate in a *gacS acnB* mutant. Alternatively, short chain acids have been suggested as the stimulus for GacS (BarA) (Chavez *et al*., 2010). Arguably, however, the signal(s) controlling the GacS sensor kinase activity has remained uncertain.

Given the intense interest in Gac/CsrA, including various mutant screens aimed at finding the signal/cue for GacS, we were surprised that aconitase mutants and their citrate accumulation had not previously been noted as affecting Gac/Csr. A plausible explanation may be that most of the bacteria used in those studies contain two or three copies of aconitase (Table S2). This functional redundancy would likely prevent mutant-based approaches from discovering a link between aconitase and Gac. Similarly, studies with aconitase mutants either reported suppressor mutations in the citrate synthase gene or deliberately inactivated *gltA* to prevent citrate accumulation (Gruer *et al*., 1997; Viollier *et al*., 2001; Li *et al*., 2008; Koziol *et al*., 2009; Baumgart *et al*., 2011). Thus, it is easy to understand how a connection between Gac/Csr regulation and blocking the TCA cycle specifically at aconitase has not been made previously.

Because aconitase requires an iron-sulfur cluster for enzymatic activity, disruption of this essential cofactor through iron limitation or direct inactivation by reactive oxygen species (ROS) can result in loss of aconitase activity and concomitant citrate accumulation. For example, in *E. coli*, oxidative inactivation of AcnB led to elevated intracellular citrate levels (Varghese *et al*., 2003). High levels of this TCA cycle intermediate would likely signal a major disturbance in central carbon metabolism. Activating the Gac/Csr regulatory cascade may allow cells to divert carbon flow away from glycolysis and increase levels of glycogenesis enzymes to allow a shift toward carbon storage until the cell is able to recover from the ROS damage and restore aconitase activity. Citrate has also been reported as an inhibitor of the enzyme pyruvate kinase (Ozaki and Shiio, 1969), which converts phosphoenolpyruvate to pyruvate, and this mechanism might further enhance the shift of carbon away from the TCA cycle.

The conditions known to affect aconitase activity have parallels in infection models. Many hosts generate ROS in response to infecting bacteria and also confront bacteria with an iron-limiting environment. If either of these conditions disrupted aconitase activity, subsequent Gac activation would explain a unifying role for Gac as both an important regulator of central carbon metabolism and host colonization. For bacteria that must switch between free-living and host-associated lifestyles, loss of aconitase activity due to ROS or low iron encountered in host tissue would signal a major environmental change. We speculate that the Gac/Csr regulatory cascade may be conserved in host-associated γ-proteobacteria to monitor for the metabolic effect(s) of losing aconitase activity.

Given the requirement of *gacA* for host colonization by *V. fischeri* and the tractability of this symbiosis (Stabb, 2006), future work in this system may uncover how this conserved and important regulatory system controls gene expression during natural symbiotic colonization. A connection of Gac/Csr to luminescence, which is a symbiotic colonization factor, has been established previously (Whistler and Ruby, 2003). Our data indicate the native LuxR-LuxI system is required for CsrA to repress luminescence (Figs 1 and S5) consistent with CsrA directly or indirectly negatively regulating *luxR* and/or *luxI*. At least two mechanisms have been proposed for such regulation: that CsrA modulates *luxR* expression (Williams *et al*., 2012) or that CsrA directly binds the *lux* operon transcript (Ballok, 2007). Ongoing studies will provide greater insight into the regulation of symbiotic bioluminescence in *V. fischeri* and the role of Gac/Csr.

Experimental procedures

Bacterial strains, media and growth conditions

Vibrio fischeri strains were all derived from the squid isolate ES114 (Boettcher and Ruby, 1990), and grown in either Luria Bertani Salt (LBS) medium (Stabb *et al*., 2001), Artificial Sea Water Tryptone (ASWT) (Septer *et al*., 2011) or Sea Water Tryptone Marine Osomolarity (SWTO) medium (Bose *et al*., 2007) at 28°C or 24°C. *E. coli* strains were grown in either Luria Bertani (LB) medium (Miller, 1992) or Brain Heart Infusion (Difco) at 37°C. Antibiotic selection for *V. fischeri* and *E. coli* strains was as described previously (Dunn *et al*., 2005). Plasmids that contained the R6Kγ origin of replication but not the ColE1 origin of replication were maintained in *E. coli* strain DH5αλpir (Dunn *et al*., 2005) and plasmid pEVS104 (Stabb and Ruby, 2002) was maintained in strain CC118λpir (Herrero *et al*., 1990). All other plasmids were maintained in *E. coli* strain DH5α (Hanahan, 1983).

Mutant and plasmid construction

Select bacterial strains and plasmids used in this study are presented in Table 2. Oligonucleotide sequences are provided in Table S3. The construction of the plasmids and engineered mutants is described in Supporting Information, and several strains and plasmids used as intermediates or tools for genetic manipulations are described in Appendix S1. For mutant construction in *V. fischeri*, mutant alleles were mobilized on plasmids into recipients by triparental mating using CC118λpir pEVS104 as a conjugative helper (Stabb and Ruby, 2002). Potential mutants were screened for allelic exchange using PCR and appropriate antibiotic resistance markers.

To isolate suppressor mutants in the *csrB* knockout mutant, strain ANS62 was streaked onto LBS plates and incubated at room temperature until large colonies became visible. These

Table 2. Key strains and plasmids used in this work.

Strains or plasmids	Relevant characteristics[a]	Source or reference
Strains		
Escherichia coli		
CC118λpir	Δ(*ara-leu*) *araD* Δ*lac74 galE galK phoA20 thi-1 rpsE rpsB argE*(Am) *recA* λ*pir*	Herrero *et al.*, 1990
DH5α	F'/*endA1 hsdR17 glnV44 thi-1 recA1 gyrA relA1* Δ(*lacIZYA-argF*) *U169deoR*(f80*dlacI*Δ(*lacZ*)*M15*)	Hanahan, 1983
DH5αλpir	λ*pir* derivative of DH5α	Dunn *et al.*, 2005
Vibrio fischeri		
ES114	Wild-type *V. fischeri*	Boettcher and Ruby, 1990
ANS28	ES114 Δ*leuCD* Δ*acnB*	This study
ANS28.1	ES114 Δ*leuCD* Δ*acnB* suppressor mutant	This study
ANS28.2	ES114 Δ*leuCD* Δ*acnB* suppressor mutant	This study
ANS28.3	ES114 Δ*leuCD* Δ*acnB* suppressor mutant	This study
ANS28.4	ES114 Δ*leuCD* Δ*acnB* suppressor mutant	This study
ANS28.5	ES114 Δ*leuCD* Δ*acnB* suppressor mutant	This study
ANS28.6	ES114 Δ*leuCD* Δ*acnB* suppressor mutant	This study
ANS29	ES114 *lacI*^{q} $P_{A1/34}$-*luxCDABEG* Δ*acnB*	This study
ANS60	ES114 Δ*gltA*	This study
ANS61	ES114 Δ*gltA* Δ*acnB*	This study
ANS62	ES114 Δ*csrB1* Δ*csrB2*	This study
ANS62.1	ES114 Δ*csrB1* Δ*csrB2 csrA*^{R6L}	This study
ANS64	ES114 Δ*gacS*; *gacS* (VF_2082) gene deletion	This study
ANS70	ES114 Δ*acnB* Δ*csrB1* Δ*csrB2*	This study
ANS73	ES114 Δ*csrB1* Δ*csrB2 lacI*^{q} $P_{A1/34}$-*luxCDABEG*	This study
ANS74	ES114 Δ*acnB* Δ*gacS*	This study
ANS79	ES114 Δ*acnB litR*::*erm*	This study
ANS81	ES114 Δ*csrB1* Δ*csrB2 csrA*^{R6L} Δ*acnB*	This study
JB19	ES114 *litR*::*erm*	Bose *et al.*, 2007
JB22	ES114 *lacI*^{q} $P_{A1/34}$-*luxCDABEG*	Bose *et al.*, 2008
JB26	ES114 Δ*acnB*; VF_2158 gene deletion	This study
JB26.1	ES114 Δ*acnB* suppressor mutant	This study
Plasmids		
pAKD702	Promoterless *lacZ*, *oriV*$_{R6K\gamma}$, *oriV*$_{pES213}$, *oriT*, Cm^R	Bose *et al.*, 2011
pAKD912	$P_{tonB/hur}$-*lacZ*; *oriV*$_{R6K\gamma}$, *oriV*$_{pES213}$, *oriT*, Kn^R	Septer *et al.*, 2011
pAS37	Δ*leuCD* allele; *oriV*$_{R6K\gamma}$, *oriV*$_{ColE1}$, *oriT*, Erm^R, Cm^R	This study
pAS79	Δ*csrB1* allele; *oriV*$_{R6K\gamma}$, *oriV*$_{ColE1}$, *oriT*, Cm^R, Kn^R	This study
pAS80	Δ*csrB2* allele; *oriV*$_{R6K\gamma}$, *oriV*$_{ColE1}$, *oriT*, Cm^R, Kn^R	This study
pAS106	Δ*gltA* allele; *oriV*$_{R6K\gamma}$, *oriT*, Cm^R	This study
pAS114	*gltA* in pVSV105; *oriV*$_{R6K\gamma}$, *oriV*$_{pES213}$, *oriT*, Cm^R	This study
pAS118	Δ*gacS* allele; *oriV*$_{R6K\gamma}$, *oriV*$_{ColE1}$, *oriT*, Cm^R, Kn^R	This study
pJLB170	pAKD702 containing the *luxR* promoter region, *oriV*$_{R6K\gamma}$, *oriV*$_{pES213}$, *oriT*, Cm^R	Bose *et al.*, 2011
pJLB171	pAKD702 containing the *luxI* promoter region, *oriV*$_{R6K\gamma}$, *oriV*$_{pES213}$, *oriT*, Cm^R	Bose *et al.*, 2011
pVSV105	*oriV*$_{R6K\gamma}$, *oriV*$_{pES213}$, *oriT*, Cm^R	Dunn *et al.*, 2006

a. Amp^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Erm^R and *erm*, erythromycin resistance; Kn^R, kanamycin resistance.

colonies were re-streaked and the suppressor mutants, including ANS62.1, were stored at −80°C. The *csrA* locus, including 210 bp upstream of the ATG start and 26 bp downstream of the TAA stop codon, from ANS62.1 was PCR-amplified, cloned and sequenced (see Supporting Information), revealing a single base change resulting in an Arg to Leu amino acid change at the sixth codon, and we named this allele *csrA*^{R6L}. The Δ*acnB* allele was moved into strain ANS62.1 (*csrB csrA*^{R6L}) to generate ANS81 (see Supporting Information).

Luminescence assays

Vibrio fischeri cultures were grown overnight in LBS medium with antibiotic to maintain plasmid selection when necessary and diluted 1:1000 into SWTO medium with supplementation as indicated, and incubated at 24°C with shaking at 200 rpm, either in aerobic shake flasks or anaerobic bottles, as indicated. At indicated time points, 0.5 ml of samples was removed and cell density (OD_{595}) was measured using a BioPhotometer (Brinkman Instruments, Westbury, NY, USA). The cuvette was shaken to aerate the sample prior to measuring luminescence using a GLOMAX 20/20 luminometer (Promega, Madison, WI, USA) with a 10 s integration setting. Luminescence values were normalized to cell density and thereby reported as specific luminescence (luminescence/OD_{595}).

ß-galactosidase reporter assays

Vibrio fischeri strains harboring plasmid-based *lacZ* reporters were grown as described above to the indicated cell density.

Cells were harvested by centrifugation and frozen overnight at −20°C. End-point ß-galactosidase assays were performed using 2-Nitrophenyl β-D-galactopyranoside (ONPG) as the substrate as described previously (Bose *et al*., 2008).

Whole-genome resequencing

Genomic DNA was isolated from wild-type ES114 and from suppressor mutants using a phenol : chloroform protocol. Cells in 3 ml of overnight culture of *V. fischeri* grown in LBS medium were pelleted, resuspended in 500 μl of $T_{50}E_{50}$ (50 mM Tris pH 7.5, 50 mM Ethylenediaminetetraacetic acid [EDTA] pH 8) and frozen at −80°C. Twenty-five microliter of fresh lysozyme solution (10 mg lysozyme per milliliter of 10 mM Tris pH 8) was added to the frozen cells, which were allowed to thaw in a room temperature water bath with gentle mixing. Cell lysate was frozen at −80°C overnight and thawed the next day in a room temperature water bath with gentle mixing. One hundred microliter of STE (0.5% sodium dodecyl sulphate [SDS] in $T_{50}E_{50}$) was added along with 35 μl of fresh proteinase K (2 mg proteinase K per milliliter DNase-free water). The solution was mixed gently and incubated at 55°C for 1 h with occasional mixing. Ten microliter RNase A (10 mg ml^{-1} in DNase-free water) was added followed by an additional 10 min incubation at 55°C. Two hundred microliter of 5 M sodium perchlorate was added followed by gentle mixing. Six hundred forty microliter Tris-equilibrated phenol : chloroform (1:1 mixture of phenol and chloroform with phenol buffered to pH 7.8–8.0) was added, mixed by inversion, and phases were separated by centrifuging at 12 800 × g for 10 min. The aqueous phase was transferred into a new tube and the phenol : chloroform extraction repeated as described above. The aqueous phase was then extracted with 600 μl of chloroform and phases separated by centrifugation at 12 800 × g for 10 min. Chloroform extraction was repeated and DNA was recovered using alcohol precipitation and washed several times with 70% ethanol. The DNA was resuspended in Tris EDTA (TE) buffer and stored at −80°C.

DNA quality and quantity were determined by measuring the ratio of absorbance at 260 nm and 280 nm, and by running DNA samples on a Tris-acetate-EDTA (TAE) agarose gel with plasmid standards. DNA was randomly sheared into ~ 200 bp fragments and the resulting fragments were used to create an Illumina library. This library was sequenced on Illumina GAIIx sequencers generating 36 bp single end reads and these reads were aligned to the *V. fischeri* ES114 reference genome. Reads were aligned with Burrows–Wheeler Aligner bwa 0.5.8a to produce a BAM (.bam) file, and variants were called with SAMtools 0.1.7 pileup, then filtered with varFilter.pl -D 300 from the SAMtools package (Li *et al*., 2008; Li and Durbin, 2009). The reads were also aligned and putative single nucleotide polymorphisms (SNPs) called using maq-0.7.1 at default settings. The two call sets were then manually combined and evaluated to produce the final list of candidate indels and SNPs.

qRT-PCR analysis

Vibrio fischeri strains were grown as described for luminescence assays to an OD_{595} of ~ 1.0. Five milliliter of culture was transferred to a chilled tube containing 1/5 volume 5% (v/v) phenol pH 4.3 (Sigma, St. Louis, MO, USA) and 95% ethanol. Cells were chilled on ice for at least 30 min and pelleted by centrifuging at 3220 × g at 4°C for 10 min. Cell pellets were stored overnight at −80°C. RNA isolation was performed using the Stratagene Absolutely RNA Miniprep Kit according to manufacturer's instructions. The RNA sample was treated with Ambion Turbo DNA-free kit to remove contaminating DNA. RNA was quantified using a Microspot Take3 plate and Synergy 2 plate reader with Gen5 software (Biotek, Winooski, VT, USA). cDNA was synthesized using Superscript II followed by an RNase treatment. The cDNA was recovered using a DNA clean/concentrate kit (Zymo Research, Orange, CA, USA), eluting with TE buffer. The cDNA was quantified as described above for the RNA. qRT-PCR reactions were performed using Sybr Green supermix (BioRad Laboratories, Hercules, CA, USA) and primers specific for either *csrB1* (AScsrB1RTF1 and AScsrB1RTR1) or *csrB2* (AScsrB2RTF2 and AScsrB2RTR2) target cDNA (for primers, see Supporting Information). CsrB copy number was determined using a standard curve with the respective target sequence cloned into pCR-BluntII-TOPO vector as template. The standard curve reactions covered a six-log dilution series and reaction efficiency for *csrB1* (98%) and *csrB2* (106%) fell within the desired range of 90–110%. Results in Fig. 8A show the average log-transformed data from three independent biological replicates per strain.

Citrate assays

Vibrio fischeri cultures were grown as indicated above for luminescence assays. At an OD_{595} of ~ 1.0, the colony-forming units (CFU) ml^{-1} was determined by dilution plating, and a 14 ml of sample was removed, chilled on ice and then centrifuged for 10 min at 15 300 × g at 4°C to pellet cells. The supernatant was removed and cells were lysed by resuspending the pellet in 350 μl of water and incubated at room temperature for 10 min. Cell lysate was centrifuged to remove cell debris and the supernatant was stored at 4°C overnight. Citrate levels in cell lysates were determined using the BioVision (Mountain View, CA, USA) citrate assay kit, following manufacturer's instructions for the fluorometric protocol and a Synergy 2 plate reader with Gen5 software (BioTek).

Estimation of ΔacnB suppression rate

To estimate the frequency of suppression of the Δ*acnB* mutant slow-growth phenotype, Δ*acnB* mutant cultures were first grown in nonselective conditions, specifically on LBS agar plates in an anaerobic jar at 28°C for ~ 24 h. Some colonies were then scraped off the plates, resuspended and dilution-plated to determine CFU per colony, and the plates were then allowed to incubate aerobically at 28°C for 2 more days. Growth of suppressor sectors on specific Δ*acnB* mutant colonies was then scored, facilitated by a low-magnification microscope. We determined the number of suppressors evident per *acnB* colony, which together with the CFU per Δ*acnB* colony determined at the start of aerobic incubation allowed us to estimate the frequency of suppressor mutant per Δ*acnB* mutant CFU.

Squid colonization assays

Colonization of *E. scolopes* by *V. fischeri* was performed essentially as previously described (Stabb and Ruby, 2003). Briefly, *V. fischeri* cultures were grown unshaken in ASWT broth at 28°C to an OD_{595} of 0.3–0.8 and then diluted in Instant Ocean (United Pet Group, Cincinnati, OH, USA) to a similar inoculum density < 3000 CFU ml^{-1} for the strains being compared. Aposymbiotic *E. scolopes* hatchlings were added to the inoculum water overnight and transferred the next morning to *V. fischeri*-free Instant Ocean. After 24 h, squid were homogenized and plated to determine CFU per squid.

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